University of Alberta

Investigation of Calreticulin-Protein Interactions

by

Christi Andrin ©

A Thesis Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of

the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

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Christi Andrin
(Author's signature)

11115 - 36 Avenue
Edmonton, Alberta
Canada
T6J 0C2

Date: January 20/99

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Investigation of Calreticulin Protein Interactions* submitted by Christi Andrin in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Dr. Marek Michalak

Dr. Larry Fliegel

Dr. Robert Hodges

Charlotte Spencer
Dr. Charlotte Spencer

Hiralal Nakhasi
Dr. Hira Nakhasi

Date: DECEMBER 17/98

ABSTRACT

Calreticulin is a conserved, calcium binding protein of the endoplasmic reticulum lumen. The protein has been implicated in several functions emphasizing its multifunctional nature. Much controversy exists as to how calreticulin can affect multiple cellular functions from possibly multiple cellular locations. This study was designed to examine the structure of calreticulin and potential calreticulin-protein interactions in hopes of furthering our understanding of how calreticulin is functioning within the cell.

Initially, the *Pichia* protein expression system was established and a purification scheme developed for the production of calreticulin for structural studies. The resulting protein was characterized and was found, unexpectedly, to be glycosylated. The glycosylation of native forms of calreticulin appears to be tissue and species specific making this finding significant. The protein was found to be antigenic upon injection into rabbits and resulted in the generation of calreticulin specific antibodies. These antibodies were characterized and their usefulness established.

Calreticulin is known to bind to the DNA binding domain of the glucocorticoid receptor *in vitro* and to be able to alter glucocorticoid sensitive gene expression *in vivo*. This interaction suggested that calreticulin may be localized to the cytoplasm or nucleus to allow for the direct modulation of steroid sensitive gene expression. The interaction between calreticulin and the glucocorticoid receptor was examined in detail. This study establishes that the direct interaction between calreticulin and the glucocorticoid is not likely of physiological relevance. As well, the ability of calreticulin to alter gene expression is confined to the protein from its endoplasmic reticulum location. Calreticulin targeted to the cytoplasm is ineffective in modulating gene expression.

Calreticulin co-localizes and co-purifies with perforin from the lytic granule of cytotoxic T-lymphocytes and therefore may interact with perforin. Calreticulin in the granules contained a C-terminal KDEL endoplasmic reticulum retrieval sequence and was targeted to the granules even in the absence of perforin. Calreticulin was found to interact directly with perforin in a calcium dependent manner both *in vitro* and *in vivo*. Therefore, calreticulin may play a dual role in cytotoxic T-lymphocytes: assist perforin in targeting to the granules and participate in cytotoxic T-lymphocyte mediated target cell killing.

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CHAPTER ONE

INTRODUCTION

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CALRETICULIN

History of Calreticulin

Calreticulin was initially identified as the high affinity calcium binding protein (HACBP) of the SR of skeletal muscle (MacLennan *et al.*, 1972; Ostwald and MacLennan, 1974). The HACBP did not begin to receive real attention until its molecular cloning in 1989 (Fliegel *et al.*, 1989b). Several other calcium-binding proteins were also discovered before and around the same time as the HACBP. CAB-63/calregulin (Waisman *et al.*, 1985), CRP55 (Koch, 1987; Macer and Koch, 1988), ERp60 (Lewis *et al.*, 1985), CaBP3 (Peter *et al.*, 1992) and a calsequestrin-like protein (Damiani *et al.*, 1988; Volpe *et al.*, 1988) were all reported as ER calcium-binding proteins. With the availability of the HACBP cDNA and its deduced amino acid sequence it quickly became clear that these investigators were all studying the HACBP or HACBP "like" proteins. To avoid the confusion of all the different names identifying the HACBP the protein was renamed calreticulin to reflect its calcium binding ability (cal-) and its localization to the ER (-reticulin).

Since the discovery of calreticulin there have been many more proteins identified that are homologous to calreticulin. A 60 kDa protein was identified as a component of the Ro/SS-A autoantigen and upon isolation of the cDNA clone was determined to be the human homologue of calreticulin (McCauliffe *et al.*, 1990a; McCauliffe, *et al.*, 1990b). A presynaptic calcium-binding protein found in *Aplysia* that was up regulated in response to long-term sensitization has 72% identity to the human calreticulin amino acid sequence

(Kennedy *et al.*, 1992). An iron binding protein, mobilferrin, was identified in rat duodenal mucosa (Conrad *et al.*, 1990) that also proved to be similar/identical to calreticulin (Conrad *et al.*, 1991). The immunization of goats with murine melanomas resulted in the production of antibodies that recognized the protein B50 (Gersten and Marchalonis, 1979). The N-terminal sequence analysis of B50 showed marked identity with calreticulin (10 out of 13 residues) (Gersten *et al.*, 1991). Johnson *et al.* (1992) isolated a protein from bovine brain they coined CBP-58 that also proved to be calreticulin. Later, it was proposed that bovine brain may contain two isoforms of calreticulin (Liu *et al.*, 1993). In addition to the original 1.9 kB transcript (Johnson *et al.*, 1992) a 3.75 kB transcript was isolated with homology to calreticuliN in the last 318 amino acids but complete divergence in the N-terminal 69 amino acids (Liu *et al.*, 1993). A protein isolated as the C1q receptor for its ability to bind to the C1 component of complement also proved to be similar to calreticulin (Malhotra *et al.*, 1993). With all the different investigators finding these calcium binding proteins it became clear that calreticulin or members of a calreticulin "like" family of proteins which may play important roles in a vast array of species and tissues.

Amino acid sequence of calreticulin

Mature rabbit calreticulin consists of 401 amino acids (Fig. 1-1). Comparison of the amino acids sequence of calreticulin isolated from various tissues and species shows a high degree of identity. From mammals to nematodes to higher plants there is a marked amino acid sequence identity between the different calreticulins isolated indicating that calreticulin is highly conserved throughout evolution. When comparing the calreticulin amino acids sequences of human (McCauliffe *et al.*, 1990a), mouse (Smith and Koch, 1989), rabbit (Fliegel *et al.*, 1989b) and rat (Murthy *et al.*, 1990) there is a greater than 90% identity between the different species. The *Aplysia* (Kennedy *et al.*, 1992) form of calreticulin is 72% identical

Figure 1.1: Rabbit skeletal muscle calreticulin amino acid sequence.

The amino acid sequence of rabbit skeletal muscle CRT is shown as deduced from the nucleotide sequence of its cDNA clone (Fliegel *et al.*, 1989a). CRT contains 401 amino acids and ends in a -KDEL ER retention/retrieval sequence as shown in bold print. The putative nuclear localization signal and putative lysosomal targeting sequences are underlined and labeled. The putative glycosylation site located at asparagine 327 is denoted by an asterisk (*). Histidine residues are indicated in bold and shadowed print and cysteine residues are just in bold print. CRT also contains a single disulfide bridge shown between cysteines 120 and 146.

Figure 1-1: Calreticulin amino acid sequence

```
         10          20          30          40          50
EPVVYFKEQF  LDGDGWTERW  IESKHKSDFG  KFVLSSGKFY  GDQEKDKGLQ
                                                  lysosomal
                                                  targeting

         60          70          80          90         100
TSQDARFYAL  SARFEPFSNK  GQPLVVQFTV  KHEQNIDCGG  GYVKLFPAGL

        110         120         130         140         150
DQKDMHGDSE  YNIMFGPDIC  GPGTKKVHVI  FNYKGKNVLI  NKDIRCKDDE
                     |_____________ S - S bridge ______|

        160         170         180         190         200
FTHLYTLIVR  PDNTYEVKID  NSQVESGSLE  DDWDFLPPKK  IKDPDASKPE
                                          NLS

        210         220         230         240         250
DWDERAKIDD  PTDSKPEDWD  KPEHIPDPDA  KKPEDWDEEM  DGEWEPPVIQ

        260         270         280         290         300
NPEYKGEWKP  RQIDNPDYKG  TWIHPEIDNP  EYSPDANIYA  YDSFAVLGLD

        310         320   *     330         340         350
LWQVKSGTIF  DNFLITNDEA  YAEEFGNETW  GVTKTAEKQM  KDKQDEEQRL
                          *Putative glycosylation site  lyso-

        360         370         380         390         401
KEEEEEKKRK  EEEEAEEDEE  DKDDKEDEDE  DEEDKDEEEE  EAAAGQAKDEL
-somal                                                ER
targeting                                          retention
```

to human calreticulin, the amino acid sequence of plant calreticulin is greater than 50% identical and the amino acid sequence of *Leishmania* is 42% homologous (Joshi *et al.*, 1996). Figure 1-2 shows a comparison of the calreticulin amino acid sequences from various species.

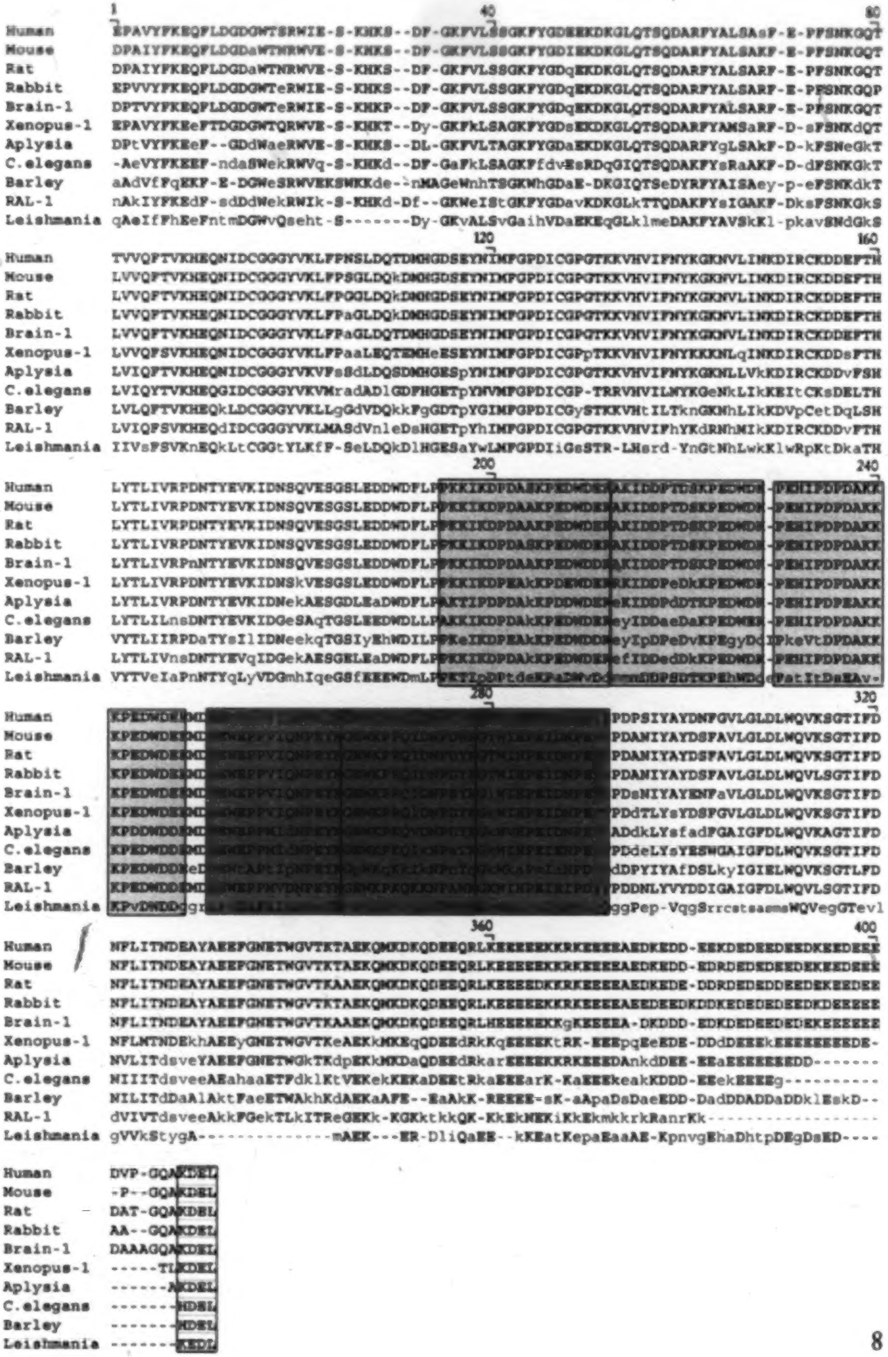
The molecular weight of rabbit calreticulin calculated from its amino acid sequence is 46,567 (Fliegel *et al.*, 1989b; Smith and Koch, 1989) but experimental determination of its apparent MW by SDS-PAGE indicated that it is a 60-63 kDa protein (Waisman *et al.*, 1985: McCauliffe *et al.*, 1990a; Milner *et al.*, 1991). The discrepancy was assumed to be due to the great number of acidic amino acid residues present in the protein.

Mature, full-length calreticulin contains 109 acidic amino acid residues, most of which are located in the carboxyl tail of the protein. This is reflected in the low pI of the protein of 4.65 - 4.67 (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990a). Calsequestrin is another example of a protein that also shows aberrant migration on SDS-PAGE that is assumed to be due to its highly acidic C-terminal region (MacLennan *et al.*, 1983).

Analysis of the calreticulin amino acid sequence revealed several important features. There is an N-terminal signal sequence targeting calreticulin to the ER lumen (Fliegel *et al.*, 1989b; Rokeach *et al.*, 1991) and a putative nuclear localization signal (amino acid residues 187-195) (Fig. 1-1). Two lysosomal targeting signals have also been found at amino acid residues 42-48 and 347-353. At this point, with the exception of the N-terminal ER targeting signal, it is unknown if these targeting signals are functional. Importantly, calreticulin was also found to contain a C-terminal K-D-E-L retention signal (Pelham, 1989) that is responsible for the retreival the ER lumnela proteins including calreticulin (Pelham, 1991; Sönnichsen *et al.*, 1994).

Figure 1.2: Calreticulin amino acid alignment

Comparison of human, mouse, rabbit, brain, *Xenopus*, *Aplysia*, *C. elegans*, barley, RAL-1 and *Leishmania* CRT amino acid sequences is shown. Conservative residues are shown in capital letters and non-conservative residues, in comparison with the human sequence, are shown in small letters. Gaps in the sequences are denoted by (-). The location of the three highly conserved amino acid repeats, PxxIPDPxAxKPEDWD, are boxed and shown in light grey. The three GxWxPPxIxNPxYx amino acid repeats are boxed and shown in dark grey. The C-terminal KDEL retention/retrieval sequence is also shown in the hatched box.



```
             1                                       40                                      80
Human        EPAVYFKEQFLDGDGWTSRWIE-S-KHKS--DF-GKFVLSSGKFYGDEEKDKGLQTSQDARFYALSAsF-E-PFSNKGQT
Mouse        DPAIYFKEQFLDGDaWTNRWVE-S-KHKS--DF-GKFVLSSGKFYGDIEKDKGLQTSQDARFYALSAKF-E-PFSNKGQT
Rat          DPAIYFKEQFLDGDaWTNRWVE-S-KHKS--DF-GKFVLSSGKFYGDqEKDKGLQTSQDARFYALSARF-E-PFSNKGQT
Rabbit       EPVVYFKEQFLDGDGWTeRWIE-S-KHKS--DF-GKFVLSSGKFYGDqEKDKGLQTSQDARFYALSARF-E-PFSNKGQP
Brain-1      DPTVYFKEQFLDGDGWTeRWIE-S-KHKP--DF-GKFVLSSGKFYGDqEKDKGLQTSQDARFYALSARF-E-PFSNKGQT
Xenopus-1    EPAVYFKEeFTDGDGWTQRWVE-S-KHKT--Dy-GKFkLSAGKFYGDsEKDKGLQTSQDARFYAMSaRF-D-sFSNKdQT
Aplysia      DPtVYFKEeF--GDdWaeRWVE-S-KHKS--DL-GKFVLTAGKFYGDaEKDKGLQTSQDARFYgLSAkF-D-kFSNeGkT
C.elegans    -AeVYFKEEF-ndaSWekRWVq-S-KHKd--DF-GaFkLSAGKFfdvEsRDqGIQTSQDAKFYsRaAKF-D-dFSNKGkT
Barley       aAdVfFqEKF-E-DGWeSRWVEKSWKKde--nMAGeWnhTSGKWhGDaE-DKGIQTSeDYRFYAISAey-p-eFSNKdkT
RAL-1        nAkIYFKEdF-sdDdWekRWIk-S-KHKd-Df--GKWeIStGKFYGDavKDKGLkTTQDAKFYsIGAKF-DksFSNKGkS
Leishmania   qAeIfFhEeFntmDGWvQseht-S-------Dy-GKvALSvGaihVDaEKEqGLklmeDAKFYAVSkKl-pkavSNdGkS
                                             120                                     160
Human        TVVQFTVKHEQNIDCGGGYVKLFPNSLDQTDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTH
Mouse        LVVQFTVKHEQNIDCGGGYVKLFPSGLDQkDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTH
Rat          LVVQFTVKHEQNIDCGGGYVKLFPGGLDQkDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTH
Rabbit       LVVQFTVKHEQNIDCGGGYVKLFPaGLDQkDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTH
Brain-1      LVVQFTVKHEQNIDCGGGYVKLFPaGLDQTDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTH
Xenopus-1    LVVQFSVKHEQNIDCGGGYVKLFPaaLEQTEMHeESEYNIMFGPDICGPpTKKVHVIFNYKKKNLqINKDIRCKDDsFTH
Aplysia      LVIQFTVKHEQNIDCGGGYVKVFsSdLDQSDMHGESpYNIMFGPDICGPGTKKVHVIFNYKGKNLLVkKDIRCKDDvFSH
C.elegans    LVIQYTVKHEQGIDCGGGYVKVMradADlGDFHGETpYNVMFGPDICGP-TRRVHVILNYKGeNkLIkKEItCKsDELTH
Barley       LVLQFTVKHEQkLDCGGGYVKLLgGdVDQkkFgGDTpYGIMFGPDICGySTKKVHtILTknGKNhLIkKDVpCetDqLSH
RAL-1        LVIQFSVKHEQdIDCGGGYVKLMASdVnleDsHGETpYhIMFGPDICGPGTKKVHVIFhYKdRNhMIkKDIRCKDDvFTH
Leishmania   IIVsFSVKnEQkLtCGGtYLKfF-SeLDQkDlHGESaYwLMFGPDIiGsSTR-LHsrd-YnGtNhLwkKlwRpKtDkaTH
                                                 200                                     240
Human        LYTLIVRPDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDASKPEDWDERAKIDDPTDSKPEDWDK-PEHIPDPDAKK
Mouse        LYTLIVRPDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAAKPEDWDERAKIDDPTDSKPEDWDK-PEHIPDPDAKK
Rat          LYTLIVRPDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAAKPEDWDERAKIDDPTDSKPEDWDK-PEHIPDPDAKK
Rabbit       LYTLIVRPDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDASKPEDWDERAKIDDPTDSKPEDWDK-PEHIPDPDAKK
Brain-1      LYTLIVRPnNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAAKPEDWDDRAKIDDPTDSKPEDWDK-PEHIPDPDAKK
Xenopus-1    LYTLIVRPDNTYEVKIDNSkVESGSLEDDWDFLPPKKIKDPEAkKPDEWDEKRKIDDPeDkKPEDWDK-PEHIPDPDAKK
Aplysia      LYTLIVRPDNTYEVKIDNekAESGDLEaDWDFLPAKTIPDPDAkKPDDWDEKeKIDDPdDTKPEDWDK-PEHIPDPEAKK
C.elegans    LYTLILnsDNTYEVKIDGeSAqTGSLEEDWDLLPAKKIKDPDAkKPEDWDEKeyIDDaeDaKPEDWEK-PEHIPDPDAKK
Barley       VYTLIIRPDaTYsIlIDNeekqTGSIyEhWDILPPKeIKDPEAkKPEDWDDReyIpDPeDvKPEgyDdIPkeVtDPDAKK
RAL-1        LYTLIVnsDNTYEVqIDGekAESGELEaDWDFLPPKKIKDPDAkKPEDWDEKefIDDedDkKPEDWDK-PEHIPDPDAKK
Leishmania   VYTVeIaPnNTYqLyVDGmhIqeGSfEEEWDmLPVKTIpDPtdeKPaDWvDdmmnEDPSDTKPEhWDdeFatItDaEAv-
                                      280                                        320
Human        KPEDWDEEMD[illegible]PDPSIYAYDNFGVLGLDLWQVKSGTIFD
Mouse        KPEDWDEKMD[illegible]PDANIYAYDSFAVLGLDLWQVKSGTIFD
Rat          KPEDWDEKMD[illegible]PDANIYAYDSFAVLGLDLWQVKSGTIFD
Rabbit       KPEDWDEKMD[illegible]PDANIYAYDSFAVLGLDLWQVLSGTIFD
Brain-1      KPEDWDEEMD[illegible]PDsNIYAYENFaVLGLDLWQVKSGTIFD
Xenopus-1    KPEDWDEKMD[illegible]PDdTLYsYDSFGVLGLDLWQVKSGTIFD
Aplysia      KPDDWDDEMD[illegible]ADdkLYsfadFGAIGFDLWQVKAGTIFD
C.elegans    KPEDWDDEMD[illegible]PDdeLYsYESWGAIGFDLWQVKSGTIFD
Barley       KPEDWDDEeD[illegible]dDPYIYAfDSLkyIGIELWQVKSGTLFD
RAL-1        KPEDWDDEMD[illegible]PDDNLYVVYDDIGAIGFDLWQVLSGTIFD
Leishmania   KPvDWDDggr[illegible]ggPep-VqgSrrcstsasmsWQVegGTevl
                                          360                                     400
Human        NFLITNDEAYAEEFGNETWGVTKTAEKQMKDKQDEEQRLKEEEEEKKRKEEEEAEDKEDD-EEKDEDEEDEEDKEEDEEE
Mouse        NFLITNDEAYAEEFGNETWGVTKTAEKQMKDKQDEEQRLKEEEEEKKRKEEEEAEDKEDD-EDRDEDEDEEDEKEEDEEE
Rat          NFLITNDEAYAEEFGNETWGVTKAAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAEDKEDE-DDRDEDEDDEEDEKEEDEE
Rabbit       NFLITNDEAYAEEFGNETWGVTKTAEKQMKDKQDEEQRLKEEEEEKKRKEEEEAEEDEEDKDDKEDEDEDEEDKDEEEEE
Brain-1      NFLITNDEAYAEEFGNETWGVTKAAEKQMKDKQDEEQRLHEEEEEKKgKEEEEA-DKDDD-EDKDEDEEDEDEKEEEEEE
Xenopus-1    NFLMTNDEkhAEEyGNETWGVTKeAEKkMKEqQDEEdRkKqEEEEKtRK-EEEpqEeEDE-DDdDEEEkEEEEEEEEDE-
Aplysia      NVLITdsveYAEEFGNETWGkTKdpEKkMKDaQDEEdRkarEEEEKKRKEEEDAnkdDEE-EEaEEEEEEEDD-------
C.elegans    NIIITdsveeAEahaaETFdklKtVEKekKEKaDEEtRkaEEEarK-KaEEEkeakKDDD-EEekEEEEg----------
Barley       NILITdDaAlAktFaeETWAkhKdAEKaAFE--EaAkK-REEEE-sK-aApaDsDaeEDD-DadDDADDaDDklEskD--
RAL-1        dVIVTdsveeAkkFGekTLkITReGEKk-KGKktkkQK-KkEkNEKiKkEkmkkrkRanrKk------------------
Leishmania   gVVkStygA--------------mAEK---ER-DliQaEE--kKEatKepaEaaAE-KpnvgEhaDhtpDEgDsED----

Human        DVP-GQAKDEL
Mouse        -P--GQAKDEL
Rat          DAT-GQAKDEL
Rabbit       AA--GQAKDEL
Brain-1      DAAAGQAKDEL
Xenopus-1    -----TLKDEL
Aplysia      ------AKDEL
C.elegans    -------HDEL
Barley       -------HDEL
Leishmania   -------KEDL
```

Cation binding to calreticulin

Calreticulin's ability to bind calcium is well established by several different laboratories (MacLennan *et al.*, 1972; Ostwald and MacLennan, 1974; Waisman *et al.*, 1985; Van *et al.*, 1989; Treves *et al.*, 1990; Milner *et al.*, 1991; Michalak *et al.*, 1991; Baksh and Michalak, 1991). Calreticulin contains two calcium binding sites (Ostwald and MacLennan, 1974; Baksh and Michalak, 1991). The protein binds calcium with a high capacity and low affinity (B_{max} = >20 moles Ca^{2+}/mole protein; K_d = 2 mM) as well as with a high affinity and low capacity (B_{max} = 1 mole Ca^{2+}/mole protein; K_d = 1 μM) (Baksh and Michalak, 1991). Calreticulin does not contain a consensus EF hand motif (Fliegel *et al.*, 1989a; Smith and Koch, 1989) found in other high affinity calcium binding proteins (Krestinger *et al.*, 1988). Calcium binding to calreticulin is inhibited in the presence of millimolar conentrations of magnesium indicating that the protein also binds magnesium with relatively low affinity *in vitro* (Baksh and Michalak, 1991). Calreticulin binds zinc with a moderate affinity (B_{max} = 14 moles Zn^{2+}/mole protein; K_d = 300 μM) (Khanna *et al.*, 1986; Khanna *et al.*, 1987). Upon zinc binding to calreticulin the protein undergoes significant conformational changes, as measured by fluorescence microscopy, that result in an increased hydrophobicity of the protein (Khanna *et al.*, 1986; Khanna *et al.*, 1987; Heilmann *et al.*, 1993). Zinc binds to the N-terminal region of calreticulin (Baksh *et al.*, 1995b). DEPC modification studies revealed that 5 out of 7 histidine residues are involved in zinc binding (Baksh *et al.*, 1995b). The functional significance of zinc binding to calreticulin is not clear.

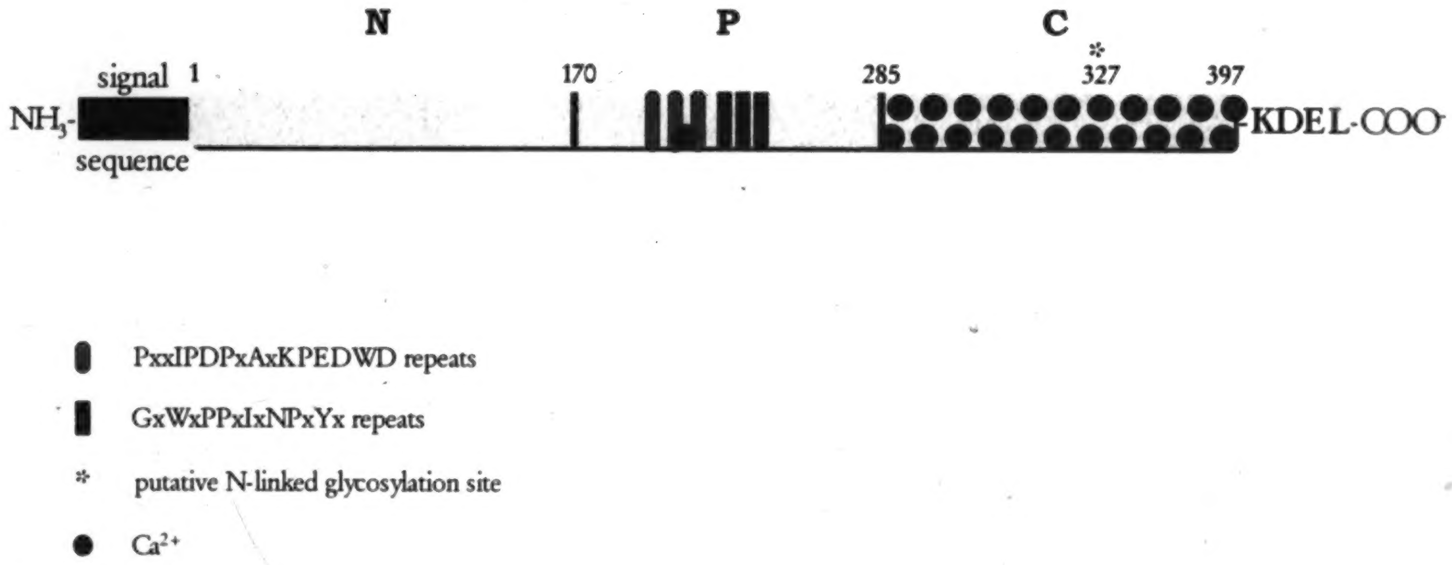
Domain structure of calreticulin

Calreticulin is predicted to contain three structural and functional domains based on its amino acid sequence (Smith and Koch, 1989; Michalak *et al.*, 1992) (Fig. 1-3). The N-terminal half of the protein has been coined the N-domain and encompasses the first 170 amino acids. It is predicted to be a highly folded, globular domain containing two short α-helicies followed by 8 anti-parallel β-strands connected by loop structures (Smith and Koch, 1989). This domain is the most highly conserved of the protein (reviewed in Michalak *et al*, 1992; Michalak ed., 1996). The N-domain, *in vitro*, interacts with the DNA binding domain of the glucocorticoid receptor (Burns *et al.*, 1994), with the α-subunit of integrins (Rojiani *et al.*, 1991; Leung-Hagesteijn *et al.*, 1994) and with rubella virus RNA (Singh *et al.*, 1994). This region of the protein contains the only three cysteine residues in calreticulin at amino acids 88, 120 and 146. The presence of a disulfide bridge between cysteine 120 and 146 has been reported (Matsuoka *et al.*, 1994).

The P-domain of calreticulin is located in the middle portion of the molecule and encompasses residues 187-285. This region was named due to its particularly high proline content. This region contains two sets of three amino acid sequence repeats (Fig. 1-1). The first three repeats are of the amino acid sequence P-X-X-I-X-D-P-D-A-X-K-P-E-D-W-D-E which is then followed by three repeats of the sequence G-X-W-P-P-X-I-X-N-P-X-Y-X. The P-domain binds calcium with high affinity calcium binding site (Baksh and Michalak, 1991). The first set of three repeats may be responsible for this calcium binding site (Michalak, unpublished results). These repeats are also found in the ER, membrane bound chaperone, calnexin, calmegin and the CALNUC protein (Wada *et al.*, 1991; Bergeron *et al.*, 1994; Lin *et al.*, 1998) implicating that these proteins may perform similar functions. Recently, the P-domain has been identified as a carbohydrate binding chaperone region in calreticulin (Vassilakos *et al.*, 1998). This region of calreticulin is also involved in the interaction between calreticulin and PDI and the modulation of PDI's function (Baksh *et al*, 1995a).

Figure 1.3: Putative calreticulin domains.

This is a schematic diagram of the putative domains of CRT as predicted from the amino acid sequence. The protein is though to be divided into three structural domains, the N-, P- and C-domains. As shown CRT contains an N-terminal signal sequence and a C-terminal ER retention/retrieval sequence. The general location of the two sets of three conserved amino acid repeats are shown by the light and dark grey rectangles. Calcium binding is depicted by the dark circles showing the high capacity binding ability of the C-domain and the low capacity of the P-domain. The putative N-linked glycosylation site as residue 327 is marked by an asterisk (*).


N
P
C
signal
sequence
1
170
285
*
327
397
NH₃-
-KDEL-COO⁻
PxxIPDPxAxKPEDWD repeats
GxWxPPxIxNPxYx repeats
* putative N-linked glycosylation site
● Ca²⁺

The C-domain of the protein covers the amino acids 286-401 and is the most variant region of the protein. It is a highly acidic region due to the presence of several negatively charged amino acids. In the last 57 amino acids there are 37 aspartate and glutamate acid residues. This domain is responsible for the low affinity, high capacity calcium binding of calreticulin (Baksh and Michalak, 1991). This domain is also involved in protein-protein interactions. The C-domain of calreticulin interacts with a set of sarcoplasmic/endoplasmic reticulum proteins (Burns and Michalak, 1993), with the vitamin K-dependent coagulation factors, Factor IX, X and prothrombin (Kuwabara et al., 1995) and prevents restenosis (Dai *et al.*, 1997).

Phosphorylation of calreticulin

Throughout calreticulin there are several putative phosphorylation sites (reviewed in Michalak *et al.*, 1992; Michalak ed., 1996) There is a cluster of possible recognition sites for protein kinase C in the N-domain (amino acid residues 17-19, 36-38, 61-63, 68-70, 79-81 and 124-126). There are also several recognition sites for casein kinase II (residues 51-54, 172-175, 178-181, 196-200, 204-208, 307-311 and 316-319) and one putative tyrosine kinase site at amino acids 261-268. Phosphorylation of calreticulin by these kinases in vitro has proved to be unsuccessful (Michalak *et al.*, unpublished observations).

Interestingly, calreticulin isolated from Vero 76 cell lysates for its ability to bind to rubella virus 3' (+) stem loop RNA is phosphorylated (Singh *et al.*, 1994). Originally, studies examining the binding of host proteins to the 3' stem loop structure of rubella virus RNA revealed a ~60 kDa protein that bound specifically to the viral RNA from both uninfected and infected Vero 76 cells (Nakhasi *et al.*, 1990; Nakhasi *et al.*, 1991). Analysis of the 60 kDa protein revealed that its phosphorylation was essential for RNA binding (Nakhasi *et al.*, 1990;

Michalak ed., 1996) and identified it as being the simian isoform of calreticulin (Singh *et al.*, 1994). Examination of the phosphorylation mechanism revealed that simian calreticulin undergoes auto-phosphorylation at both serine and threonine residues (Atreya *et al*, 1995). Calreticulin binding to viral RNA is increased in rubella virus infected cells even though its steady state protein levels appear the same in uninfected and infected cells (Nakhasi *et al.*, 1990; Michalak ed., 1996). The increased RNA binding is attributed to the hyperphosphorylation of calreticulin induced by the rubella virus infection of the cells (Nakhasi *et al.*, 1990). *Leishmania* calreticulin is another example that binds RNA and is phosphorylated by an auto-kinase mechanism (Joshi *et al.*, 1996). At present these are the only phosphorylated forms of calreticulin that have been detected.

Glycosylation of calreticulin

Calreticulin contains a putative N-linked glycosylation site at amino acid 327 (Fig. 1-1). However, no glycosylation of calreticulin has been detected in mouse, rabbit pancreas or liver, rat sperm, dog pancreas or chicken liver proteins (Michalak ed., 1996). On the other hand, bovine brain, bovine liver, rat liver, Chinese Hamster Ovary cell, spinach and *Leishmania* calreticulin (Waisman *et al.*, 1985; Van *et al.*, 1989; Jethmalani *et al.*, 1994; Matsuoka *et al.*, 1994; Joshi *et al.*, 1996; Navazio *et al.*, 1996) have been reported to be glycosylated. Calreticulin isolated from bovine brain and from *Schistosoma mansoni* have an additional glycosylation site at residue 162 (Matsuoka *et al.*, 1994). The C1q receptor, a homologue of calreticulin, is also glycosylated (Malhotra *et al.*, 1993). More recently, glycosylation of human calreticulin in the myeloid HL-60 cell line and in Sf9 insect cells was investigated (Denning *et al.*, 1997). The glycosylation of the human calreticulin was not detectable by conventional endoglycosidase digestion but was detectable with a more sensitive oxidative derivitization of the carbohydrate chain (Denning *et al.*, 1997).

Another circumstance where calreticulin appears to be glycosylated is in Chinese hamster ovary cells subjected to heat stress (Jethmalani *et al.*, 1994a) and amino acid deprivation (Heal and McGivan, 1998). Cells subjected to heat stress undergo a process called "prompt protein glycosylation" whereby a set of proteins are glycosylated rapidly after heat stress (Henle *et al.*, 1993; Henle *et al.*, 1995). Calreticulin was identified as one of those proteins (Jethmalani et al., 1994a). Glycosylation of calreticulin under these conditions is independent of both new protein synthesis (Jethmalani *et al.*, 1994a) and changes in calcium homeostasis (Jethmalani and Henle, 1994b).

Regulation of expression of the calreticulin gene

Analysis of the mouse calreticulin gene revealed several putative transcriptional control elements (Waser *et al.*, 1997). Two AP-1 sites were identified (nucleotides -1,034 and -1,378), eight AP-2 sites (nucleotides -74, -258, -300, -305, -518, -553, -1,091, -1,098, -1,251 and -1,477), three SpI sites (nucleotides -76, -303 and -312), four CCAAT sequences (nucleotides -194, -207, -1,123 and -1,532) and a TATA box (nucleotides -20 to -25) (Waser *et al.*, 1997). Also, a single H4TF-1 site and a SIF PDGF binding site were located at nucleotides -183 and -404, respectively (Waser *et al.*, 1997). The comparison of the mouse gene (Waser *et al.*, 1997) with the human gene (McCauliffe *et al.*, 1992) revealed a 70% identity between the two nucleotide sequencesindicating the conserved nature of calreticulin as well as suggesting that they may be under similar transcriptional control mechanisms.

The expression of calreticulin is affected by different conditions and cellular events in several different cell lines. Amino acid deprivation leads to increased expression of calreticulin in both bovine kidney NBL-1 cells (Plakidou-Dymock and McGivan, 1994) and in Chinese hamster ovary cells (Heal and McGivan, 1998). Nguyen *et al.* (1996) and Waser *et al.* (1997) also showed a significant increase in calreticulin gene expression in response to calcium store depletion. Treatment of HeLa cells with ionomycin or thapsigargin resulted in a marked increase in calreticulin mRNA levels indicating that calcium store depletion causes an activation of calreticulin gene expression (Llewllyn *et al.*, 1996). The detectable increase in gene expression was confirmed to be at the transcription level by nuclear run-on experiments and was found to beindependent of the extracellular calcium concentration (Waser *et al.*, 1997). The resulting increase in protein levels were also inhibited by cyclohexamide indicating the requirement for new protein synthesis (Waser *et al.*, 1997). Deletion analysis of the mouse calreticulin promoter revealed two regions (nucleotides -115 to -260 and -685 to -1,763) responsible for the induction of calreticulin expression in response to calcium store depletion (Waser *et al.*, 1997). The calreticulin promoter is also activated by zinc (Nguyen *et al.*, 1996). Cellular events such as proliferation (Opas et al., 1991), T-cell stimulation (Burns et al., 1992), viral infection (O'Baanion *et al.*, 1993; Zhu and Newkirk, 1994) and *Leishmania* parasite differentiation (Joshi *et al.*, 1996) also result in an increase in calreticulin levels.

Heat shock is another important event that causes an increase in calreticulin expression (Conway *et al.*, 1995; Szewczenko-Pawlikowski *et al.*, 1997). Heat shock results in a stress response that alters the cells physiology (Morimoto, 1993; Morimoto, 1994) and cells respond by increasing expression of a family of heat shock proteins as well as producing higher levels of the endogenous molecular chaperones (Ellis, 1991; Morimoto, 1993; Morimoto, 1994). The expression of these proteins are under the control of a heat shock element (HSE) found in their respective promoters which is bound by a protein known as the heat shock factor (HSF) (Morimoto, 1993). The shortest stretch of DNA that the HSF has been found to bind is nGAAnnTTCn (Fernandes *et al.*, 1994) and a similar such sequence has been identified in the 5' flanking region of the calreticulin gene (5'-

gGAAccCAGcgTTC-3') (Conway *et al.*, 1995; Szewczenko-Pawlikowski *et al.*, 1997). It was suggested that calreticulin's elevated expression in response to heat shock may be regulated by the HSF like other heat shock proteins (Conway *et al.*, 1995).

Tissue distribution and cellular localization of calreticulin

Calreticulin is a ubiquitous protein since it has been found in a wide variety of cells examined from mammals to nematodes to unicellular parasites (Michalak ed., 1996; Nakhasi *et al.*, 1998). Calreticulin has also been identified in plants (Menegazzi *et al.*, 1993; Opas *et al.*, 1996). Calreticulin is found in many different species it has been found in many different tissues (Khanna and Waisman, 1986; Fliegel *et al.*, 1989a; Treves *et al.*, 1990). The protein is more abundant in pancreas, liver and testis at 200 - 500 µg/g of tissue than in kidney, spleen, adrenals and parathyroid which only had approximately 100 µg/g of tissue (Khana and Waisman, 1986). The presence of calreticulin in tissues such as the cerebral cortex and muscle tissues was very low (~ 20 µg/g of tissue) (Khana and Waisman, 1986). The only eukaryotic cells that calreticulin has not been found in are yeast and erythrocytes (Khanna and Waisman, 1986).

Several researchers over the years have examined calreticulin from many different sources and have well established calreticulin as a ER lumenal protein (Ostwald and MacLennan, 1974; Michalak *et al.*, 1980; Lewis *et al.*, 1985; Lewsi *et al.*, 1986; Koch and Macer, 1988; Fliegel *et al.*, 1989a, 1989b; Smith and Koch, 1989; Krause *et al.*, 1990; Treves *et al.*, 1990; Michalak *et al.*, 1991; Milner *et al.*, 1991; Opas *et al.*, 1991; Perrin *et al.*, 1991; Tharin *et al.*, 1992; Parys *et al.*, 1994; Sönnichesen *et al.*, 1994; Copray *et al.*, 1996; Opas *et al.*, 1996). Calreticulin's localization to the ER is not a great surprise due to its N-terminal ER signal sequence and its C-terminal K-D-E-L retention signal (Fliegel *et al.*, 1989b). It is assumed that if calreticulin escapes the ER its K-D-E-L retrieval signal will be recognized by the 22 kDa K-D-E-L receptor and retrieved back to the ER (Lewis and Pelham, 1990; Lewis and Pelham 1992; Sönnichesen *et al.*, 1994). There is evidence that both retention and retrieval mechanism contribute to calreticulin's localization to the ER and that this localization is not altered by overexpression of the protein (Sönnichesen *et al.*, 1994).

Calreticulin does manage to become localized to different cellular compartments under some circumstances (Meldolesi *et al.*, 1996). A calcium binding protein was isolated from rat spermatogenic cells (Nakamura *et al.*, 1991) and later identified as calreticulin (Nakamura *et al.*, 1993). Immunostaining and immunoelectron microscopic studies of the cells revealed that calreticulin was localized to the acrosome of spermatids and sperm as well as in the Golgi apparatus (Nakamura *et al.*, 1993). Examination of the deduced amino acid sequence showed that the calreticulin isolated from spermatogenic cells did contain a K-D-E-L retention signal (Nakamura *et al.*, 1993). The isolated protein was not glycosylated and bound calcium with both high and low affinities like calreticulin isolated from other sources (Nakamura *et al.*, 1993). Calreticulin therefore, escapes the ER retention/retrieval mechanism and may be involved in spermatogenesis. Calreticulin was also found localized to the cytotoxic T-cell granules by immunocytochemistry and by its co-purification with perforin from isolated granules (Dupuis et al., 1993) (discussed in Chapter 5).

Calreticulin is also found in extracellular locations implying its secretion from these cells. There are low levels of calreticulin in human serum (Sueyoshi *et al.*, 1991). The protein has anti-thrombotic capabilities likely through its ability to bind blood coagulation factors IX, X and prothrombin (Kuwabara *et al.*, 1995). Calreticulin has been identified on the surface of red blood cells along with other ER proteins from patients with congenital dyserythropoietic anemia (Texier *et al.*, 1996). Calreticulin on the surface of human fibroblast interacts with fibrinogen(Gray *et al.*, 1995). As well, cell surface calreticulin bound to carbohydrate was identified from B16 mouse melanoma cells (White *et al.*, 1995). Calreticulin also binds to glycosylated laminin (McDonnell *et al.*, 1996). This may be responsible for the protein's ability to trigger cell spreading (White *et al.*, 1995). One of the most interesting extracellular locations of calreticulin is in the saliva of ticks although its role in the feeding process of ticks is unknown (Jaworski *et al.*, 1995).

Calreticulin is released from activated neutrophils and then binds to the C1q component of complement (Eggleton *et al.*, 1994; Kishore *et al.*, 1997). C1q binding provides the initial activation of the complement cascade (Cooper, 1985). The N-domain of calreticulin interacts with C1q and through that interaction may inhibit some of the functions of the classical complement pathway (Kishore *et al.*, 1997). A calreticulin homologue, cC1q-R, is also released from activated Raji (lymphoma) cells or peripheral blood lymphocytes (Peterson *et al.*, 1997). As well, evidence for the extracellular localization of calreticulin comes from calreticulin being found in the serum of patients with systemic lupus erythramatosus or celiac disease and its ability to act as an autoantigen (Eggleton *et al.*, 1997; Tu ková *et al.*, 1997).

There has been suggestions that calreticulin may be localized to the cytoplasm due to the fact that calreticulin has been found to be able to interact with the α subunit of integrins and modulate their function (Rojiani *et al.*, 1991; Coppolino *et al.*, 1995). Calreticulin's ability to interact with various steroid hormone receptors and affect steroid sensitive gene expression has also indicated that calreticulin may, at some point, gain access to the cytoplasm (Burns *et al.*, 1994a; Burns *et al.*, 1994b; Dedhar *et al.*, 1994; Winrow *et al.*, 1995; Desai *et al.*, 1996; Shago *et al.*, 1997). There is also the possiblilty that calreticulin may interact with these steroid receptors in the nucleus. Calreticulin does contain a putative nuclear localization signal but has only been detected in the nucleus of COS cells (Roderick *et al.*, 1997). Calreticulin has been specifically located to the nucleoli of some cells (Opas *et al.*, 1991; Opas and Michalak, 1992) although the significance of this localization is not clear. There is suspicion that the nuclear calreticulin detected is due to an artifact since the immunocytochemically detected nuclear calreticulin antigen has only been identified in cells of rat origin (Michalak ed., 1996). There is the possibility that a protein containing a similar epitope to calreticulin exists in the nucleus of these cells.

Although the possibility that calreticulin may gain access to the cytoplasm has been implied, no direct proof has been found to date of the protein at that location. It has been assumed that if calreticulin is localized to the nucleus that it likely gains entry to the nucleus through the cytoplasm. It is possible that calreticulin is only found in the cytoplasm transiently or in very low amounts that have not been detected thus far but further study will need to be done to examine whether calreticulin is located in the cytoplasm or the nucleus.

Calreticulin and chaperone function

The sequence similarity between calreticulin and calnexin led several researchers to explore the possibility that calreticulin may be acting as a molecular chaperone calnexin. Calnexin is a type I integral membrane protein of the ER that acts as a molecular chaperone for newly synthesized glycoproteins (Ou *et al.*, 1993; Bergeron *et al.*, 1994; Hammond *et al.*, 1994; Hammond and Helenius, 1994; Jackson *et al.*, 1994; Rajogapalan *et al.*, 1994). Very quickly after it was established that calreticulin can also behave as a molecular chaperone by transiently interacting with nascent glycoproteins in the ER (Nauseef *et al.*, 1995; Peterson *et al.*, 1995; Wada *et al.*, 1995).

N-linked glycosylation of proteins in the ER involves the transfer of a $Glc_3Man_9GlcNAc_2$ chain (Fig. 1-3) to the asparagine in the consensus sequence N-x-S/T (for review see Helenius *et al.*, 1997 and references therein). The mature protein, upon exit of the ER, no longer contains its three terminal glucose residues. As the glycosylated protein moves through the ER glucosidase I and II sequentially remove the terminal glucose residues. It was clearly determined through the use of glucosidase inhibitors that calreticulin's interaction with the newly synthesized glycoproteins, similar to calnexin (Bergeron *et al.*, 1994; Hebert *et al.*, 1995b; Allen and Bulluid, 1997; Helenius *et al.*, 1997), was dependent on the monoglucosylated form of the carbohydrate chain (Peterson *et al.*, 1995; Wada *et al.*, 1995; Otteken and Moss, 1996; Spiro *et al.*, 1996; Wada *et al.*, 1997; Zhang *et al.*, 1997). Further investigation led to the determination that deglucosylation of the terminal glucose residue was important in the release of major histocompatability complex - β_2 microglobulin complexes from calreticulin (Van Leeuwen and Kearse, 1996) and likely for the dissociation from other glycoproteins. As a part of the quality controls mechanisms in the ER, UDP-glucose: glycoprotein glucosyltransferase senses the folding state of glycoproteins passing through the ER and selectively reglucosylates incompletely or misfolded proteins (Sousa *et al.*, 1992; Trombetta and Parodi, 1992). By re-adding the terminal glucose residue back onto the glycoprotein it can again interact with calnexin or calreticulin to promote the continuation of proper folding (Hammond and Helenius, 1993; Hebert *et al.*, 1995a; Hebert *et al.*, 1996; Van Leeuwen and Kearse, 1997; Wada *et al.*, 1997) and a cycle of chaperone assisted folding then occurs until the protein is ready to leave the ER. There is evidence that calreticulin and calnexin can interact with the monoglucosylated plasminogen activator but this interaction is not required for the proper folding of the protein to its native conformation (Allen and Bulleid, 1997). On the other hand, there is evidence that in the absence of calreticulin and calnexin binding, maturing glycosylated insulin receptors oligomerize prematurely and misfold without the assistance of the lectin like chaperones (Bass *et al.*, 1998). Regardless, the cell contains a sophisticated and complex quality control mechanism to ensure proper folding of proteins destined for cellular locations beyond the ER.

Calreticulin has been found to interact with both cellular and viral glycoproteins in Chinese hamster ovary cells (Peterson *et al.*, 1995) but has also shown the ability to interact with mainly denatured and few native proteins extracted from human placenta or present in human serum (Wiuff and Houen, 1996). Calreticulin's ability to interact with the denatured proteins is cation dependent (Wiuff and Houen, 1996). Specifically, the interaction required the presence of calcium or magnesium and was assumed because of the high capacity binding of calcium to the C-domain of calreticulin that the ion binding resulted in a necessary conformational change to allow for binding (Wuiff and Houen, 1996). Vassilakos *et al.* (1998) determined that the interaction between calreticulin and newly synthesized glycoproteins required the presence of calcium. They also reported that the P-domain of calreticulin as being essential for the interaction with the glycan (Vassilakos *et al.*, 1998). Calreticulin is also reported to interact with radiolabeled peptides translocated into microsomes by the transporter associated with antigen processing (TAP) (Spee and Neefjes, 1997). This interaction is believed to occur through a protein complex between calreticulin, major histocompatablity complex-β_2 microglobulin heterodimers and TAP supporting calreticulin's involvement in the folding and assembly of protein complexes in the ER (Spee and Neefjes, 1997).

There has been confusion as to why the ER might contain what appeared to be two redundant molecular chaperones. Calreticulin and calnexin may work together due to the fact that ternary complexes between calreticulin, calnexin and substrate have been identified (Otteken and Moss, 1996; Tatu and Helenius, 1997). There have also been complexes between newly synthesized glycoproteins, calnexin and another ER protein ERp57 (Zapun *et* *al.*, 1998). Calreticulin interacts with PDI in a calcium dependent manner (Baksh *et al.*, 1995a; Corbett *et al.*, 1998) and a zinc dependent interaction between calreticulin and ERp57 has very recently been identified (Corbett *et al.*, 1998). Some studies have indicated that calreticulin and calnexin may have some similar substrates but they also show differences in their substrate specificities (Wada *et al.*, 1995; Hebert *et al.*, 1996; Otteken and Moss, 1996; Van Leeuwen and Kearse, 1996; Hebert *et al.*, 1997; Nauseef et al., 1998; Pipe et al., 1998; Zhang and Salter, 1998). Therefore, there is convincing evidence starting to mount that calreticulin and calnexin may have separate but overlapping chaperone functions.

There are differences in the number of glycans that calreticulin and calnexin recognize (Hebert *et al.*, 1997; Zhang and Salter, 1998) but their recognition of the glycan chain itself appears to be the same (Vassilakos *et al.*, 1998). It has been determined that calreticulin and calnexin require more than simply a terminal glucose residue for binding (Spiro *et al*, 1996; Vassilakos *et al.*, 1998). It was clearly demonstrated that the truncated $Glc_1Man_4GlcNAc$ could no longer bind to calreticulin (Spiro *et al.*, 1996). Therefore, not only is a terminal glucose required. A minimum of five mannose residues, which includes the α 1-6 branch of the oligosaccharide chain, are required for calreticulin or calnexin to properly recognize and bind to the carbohydrate chain (Spiro *et al.*, 1996; Vassilakos *et al.*, 1998). The affinity of calreticulin and calnexin for various altered glycans was also determined to be the same (Vassilakos *et al.*, 1998). This indicates that the difference substrate specificities for calreticulin and calnexin are not due to the oligosaccharide structure but instead may involve protein-protein interactions. This is supported by earlier studies showing the involvement of protein-protein interactions between major histocompatability complex class I and II heavy chains with calnexin (Arunachalam and Cresswell, 1995; Williams, 1995; Zhang *et al*, 1995).

Calreticulin and cell adhesion

Calreticulin is implicated to play a role in cell adhesion (Leung-Hagesteijn *et al.*, 1994; Coppolino *et al.*, 1995; Opas *et al.*, 1996). Integrins are the most studied cell surface receptors involved in cell adhesion (Ruoslahti, 1991; Hynes, 1992). Integrins, upon ligand binding, cluster and form a focal contact (Schwartz *et al.*, 1991; McNamme *et al.*, 1993; Yamada and Miyamoto, 1995). These focal contacts are involved in adhesion of cells to the extracellular matrix (Burridge *et al.*, 1988). By deletion analysis of truncated integrin subunits, the K-x-G-F-F-K-R motif found in α integrins appears essential for the regulation of the affinity state of the integrin for its ligand (Ylänne *et al.*, 1993; O'Toole *et al.*, 1994). This K-x-G-F-F-K-R motif is also found in the DNA binding domain of steroid receptors (Laudet *et al.*, 1992). It was determined *in vitro* that calreticulin interacts with this motif in both α integrins (Rojiani *et al.*, 1991) and in steroid receptors (Burns *et al.*, 1994a; Dedhar *et al.*, 1994). Furthermore, down regulation of calreticulin expression resulted in a subsequent decrease in cellular adhesion to extracellular matrix components (Leung-Hagesteijn *et al*, 1994; Coppolino *et al.*, 1995). This inhibition of adhesion, due to decreased calreticulin expression, was determined to be integrin mediated (Leung-Hagesteijn *et al.*, 1994). The inhibition of adhesion was not detected in cells overexpressing calreticulin (Leung-Hagesteijn *et al.*, 1994). Calreticulin also co-imunoprecipitates with integrins from Jurkat cells (Coppolino *et al.*, 1995). This interaction was demonstrated to be dependent on the activation state of the integrin and to have functional consequences (Coppolino *et al.*, 1995). Treatment of streptolysin O-permeablized Jurkat cells with calreticulin specific antibodies resulted in a subsequent decrease cell adhesion (Coppolino *et al.*, 1995). It was proposed that calreticulin may play a role in cell adhesion via its ability to interact with α integrin subunits, possibly by stabilizing the activated state of the integrin (Rojiani *et al.*, 1991; Dedhar, 1994;

Leung-Hagesteijn *et al.*, 1994; Coppolino *et al.*, 1995). The question still remains as to how calreticulin escapes the ER to interact with α integrins located at the plasma membrane. Importantly, calreticulin's overexpression has not caused calreticulin to become localized to the cytoplasm (Sönnichsen *et al.*, 1994; Mery *et al.*, 1996; Opas *et al.*, 1998).

Vinculin is another molecule involved in cell adhesion (Otto, 1990) in both focal contacts and in cell-cell contacts known as zonulae adherens (Franke *et al.*, 1988). Recent studies on the role of calreticulin in cell adhesion indicate that calreticulin may be involved in modulating cell adhesion via its regulation of vinculin expression (Opas *et al.*, 1996). The overexpression of calreticulin resulted in an increase in both cell-cell and cell-extracellular matrix adhesiveness (Opas *et al.*, 1996). It was also found that increased calreticulin expression had affects on cell motility and cell spreading which are directly linked to cell adhesion (Opas *et al.*, 1996). Importantly, the downregulation of calreticulin expression had the opposite effect (Opas *et al.*, 1996). Calreticulin's involvement in cell adhesion and interaction with α integrins has implied that calreticulin may be localized to the cytoplasm although direct evidence is lacking. Calreticulin has not been found in the cytoplasm and therefore it is likely that its ability to affect cell adhesion an indirect modulation of vinculin expression due to the ER localized calreticulin (Opas *et al.*, 1998).

Calreticulin and calcium homeostasis

Calcium is an essential second messenger involved in a wide variety of cellular pathways and processes (Carafoli, 1987). Strict homeostasis of calcium levels is then required to maintain proper functioning of the cell. One of the ways that the cell controls intracellular calcium levels is by calcium binding proteins that buffer the calcium content within the cell and within cellular organelles. Calreticulin has been found to play an important role in calcium homeostasis in a variety of cells (Liu *et al.*, 1994; Bastianutto *et al.*, 1995; Camacho and Lechleiter, 1995; Mery *et al.*, 1996; Coppolino *et al.*, 1997; Fasalato *et al.*, 1997; John *et al.*, 1998). Liu *et al.* (1994) demonstrated that by reducing the level of calreticulin by using antisense oligonucleotide treatment the calcium storage capacity of mouse neuroblastoma X glioma NG-108-15 cells was decreased. It was also determined that reducing the calreticulin levels in differentiated NG-108-15 cells induces apoptosis implying that calreticulin confers resistance against apoptosis in these cells (Liu *et al.*, 1994; Johnson *et al.*, 1998). Overexpression of calreticulin also inhibits repetitive calcium wave formation in *Xenopus* oocytes in response to IP_3 (Camacho and Lechleiter, 1995). This indicated that calreticulin may be modulating the function of the IP_3 receptor or possibly inhibiting calcium uptake by the sarcoplasmic/endoplasmic reticulum ATPase calcium pump, SERCA 2b (Camacho and Lechleiter, 1995). Recently, John *et al.* (1998) have determined that a putative N-linked glycosylation site in the lumenal C-terminal tail of SERCA 2b is required for calreticulin's ability to inhibit IP_3 induced repetitive calcium waves in SEARCA 2b overexpressing oocytes. This then indicates that calreticulin's ability to alter calcium homeostasis may be because of an ability to modulate SERCA 2b function but does not excluded a possible affect on the IP_3 receptor as well (John *et al.*, 1998). In HeLa cells and in L cells calreticulin overexpression was found to both increase the capacity of the intracellular calcium stores but to also play a role in store-operated calcium influx across the plasma membrane (Basstianutto *et al.*, 1995; Mery *et al.*, 1996).

Store operated calcium influx (also known as calcium release-activated calcium current, I_{CRAC}) is activated by the depletion of intracellular calcium stores which is part of the calcium homeostasis mechanisms regulating various cellular processes (Putney, 1990; Berridge, 1995). Calreticulin overexpression results in a decrease in store operated calcium influx (Mery *et al*, 1996). ER localized calreticulin is therefore capable of affecting cellular activity occurring at the plasma membrane. Bastianutto *et al.* (1995) determined that the store operated influx of calcium was inversely correlated to the level of free calcium in the ER lumen. It has also been determined that the store activated calcium influx is solely dependent on the reduction of calcium within the ER and the kinetics involved are rather slow (Hofer *et al.*, 1998). Calreticulin's role in store operated calcium influx in somewhat controversial. Recent work by Fasolato *et al.* has shown that calreticulin overexpression has no effect on store operated calcium influx in response to rapid, complete depletion of calcium from the ER lumen. They also demonstrated that upon passive depletion of calcium from the ER that calreticulin overexpression only delayed store operated calcium influx but did not inhibit it and that this delay was dependent on the C-domain of calreticulin (Fasolato *et al.*, 1998). As well, store operated calcium influx in calreticulin knockout embryonic stem cells appears unaffected (Coppolino *et al.*, 1997). However, calreticulin was found to be essential for integrin-mediated calcium influx (Coppolino *et al.*, 1997). Increases in cytosolic calcium levels that normally accompany integrin activation were absent in calreticulin knockout embryonic stem cells (Coppolino *et al.*, 1997). Although the data surrounding calreticulin's exact role in calcium homeostasis is conflicting it is not necessarily mutually exclusive. Possibly, when the lumenal calcium stores are only partially depleted calreticulin acts to inhibit the store operated calcium influx to aid in maintaining calcium homeostasis. Upon rapid, more complete depletion of the calcium stores the signal activating the I_{CRAC} channel may be maximal and calreticulin's inhibitory effect no longer seen. Therefore, calreticulin may be involved in the calcium storage capacity of the ER calcium store but also may act as a calcium sensor or signal for store operated calcium influx across the plasma membrane. Importantly, ER localized calreticulin appears capable of affecting calcium homeostasis in different subcellular locations implicating it as a possible ER signaling molecule.

Other functional roles of calreticulin

Calreticulin has been implicated in several other functions throughout the cell. As mentioned earlier calreticulin has been identified as a stress protein that is induced in response to heat shock (Conway *et al.*, 1995; Szewczenko-Pawlikowski *et al.*, 1997) as well as amino acid deprivation (Plakidou-Dymock and McGivan, 1994; Heal and McGivan, 1998) and chemical treatments that alter calcium handling within the cell (Llewllyn *et al.*, 1996; Nguyen *et al.* 1996; Waser *et al.* 1997). Recently, it has been demonstrated that induction of ER stress proteins, including calreticulin, confers tolerance to LLC-PK1 renal epithelial cells against oxidant induced calcium increases and subsequent cell death (Liu *et al.*, 1997; Liu *et al.*, 1998). Calreticulin expression is also induced in response to viral infection (O'Baanion *et al.*, 1993; Zhu and Newkirk, 1994; Singh *et al.*, 1994) and has been found to bind to rubella virus RNA (Singh *et al.*, 1994). Calreticulin isolated from *Leishmani* has also been shown to bind to endogenous RNA (Joshi *et al.*, 1996). Calreticulin's binding to RNA is phosphorylation dependent (Nakhasi *et al.*, 1990; Michalak ed., 1996). As well, the detection of calreticulin-RNA complexes coincides with the onset of detectable negative strand RNA synthesis (Nakhasi *et al.*, 1990). This implicates calreticulin in playing a potential role in viral RNA synthesis. Presently, calreticulin's role in binding RNA is not clear but may be involved in RNA metabolism (Nakhasi *et.al.*, 1998).

Calreticulin has also been identified as an autoantigen present in the serum from patients with systemic lupus erythramatosus and with Sjögren's syndrome (McCauliffe *et al.*, 1990a; McCauliffe *et al.*, 1990b). It has been shown that immunization of a single Ro/SS-A component causes the spread of autoimmunity to the other Ro/SS-A components (Topfer *et al.*, 1995) The immunization of mice with either Ro52 or Ro60 caused the production of autoantibodies to calreticulin supporting the finding that calreticulin is an autoantigen. Anti-gliadin antibodies in cross react with calreticulin and calreticulin levels are elevated in the serum of celiac disease patients implying a role for calreticulin as an autoantigen (Tu ková*et al.*, 1997). This suggests that calreticulin may play an important role in the immune system. Another indication that calreticulin may be involved in some aspect(s) of the immune system is the finding that calreticulin expression was elevated in stimulated T-cells (Burns *et al.*, 1992) and is a component of the cytotoxic T-cell granules (Dupuis *et al.*, 1993) (discussed further in Chapter 5).

Another very important finding was that calreticulin was able to bind to the DNA binding domain of various steroid receptors (Burns *et al.*, 1994; Dedhar *et al.*, 1994; Wheeler *et al.*, 1995; Winrow *et al.*, 1995; Desai *et al.*, 1996). As well as calreticulin being able to bind to the various steroid receptors it was clearly demonstrated that calreticulin has the ability to modulate steroid sensitive gene expression (Burns *et al.*, 1994; Dedhar *et al.*, 1994; Wheeler *et al.*, 1995; Desai *et al.*, 1996) (discussed further in Chapter 4).

CHAPTER TWO

EXPERIMENTAL PROCEDURES

MATERIALS

Molecular biological reagents

Restriction endonucleases and DNA modifying enzymes were obtained from Boehringer Mannheim, Gibco/BRL Life Technologies (Burlington, Ontario) and New England Biolabs. All reactions were carried out in the buffers provided by the manufacturer. Vent polymerase was purchased from New England Biolabs. Plasmid purification kits were purchased form Qiagen Inc. (Chatsworth, CA)

Secondary antibodies

FITC and Texas Red-conjugated antibodies were from Bio/Can Scientific or ICN ImmunoBiologicals (Montreal, Quebec). Horseradish peroxidase conjugated rabbit anti-goat and goat anti-rabbit secondary antibodies were from Bio-Rad (Hercules, CA) and Boehringer Mannheim (Laval, Quebec), respectively.

Protein expression, separation, detection and characterization

Pichia expression system was purchased from Invitrogen. Lyticase, polyethylene glycol 4000 (PEG), sorbitol, histidine and Ponceau S were purchased from Sigma. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents, molecular weight markers and the DC protein concentration determination kit were from Bio-Rad. Nitrocellulose membrane filters and ECL detection kit for Western blotting were from Amersham (Oakville, Ontario). Mono Q and Resource Q FPLC columns, Cyanogen bromide (CNBr)-activated Sepharose, Glutathione-Sepharose 4B and DEAE-Sephadex G50 were all from Pharmacia (Baie d'Urfe, Quebec) The T7 TNT® Coupled Reticulocyte Lysate protein expression system was from Promega (Madison, WI). Protease inhibitors referred to as "SL inhibitors" throughout the remainder of the document were obtained from Boehringer

Mannheim. This cocktail was prepared as a 2000 x concentrated stock in 100% ethanol and was used at the following final concentrations: 0.01 mg/mL of aprotonin, phosphoramidone, 1-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, L-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, (4-amidophenyl) methanesulfonyl fluoride and N-[N-(1-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl] agmatine, 0.05 mg.mL leupeptin and 0.01 mg/mL pepstatin. Triton X-100, Tween 20, Nonident P-40, sodium deoxycholate (DOC) and horseradish peroxidase conjugated Concanavalin A (ConA) were from Sigma. Endoglycosidase F (endo F) and endoglycosidase H (endoH) modifying enzymes were both from Boehringer Mannheim. X-ray film was from Kodak (Rochester, New York).

Radiochemicals

[^{35}S]-methionine, [^{45}Ca]Cl$_2$ and γ[^{32}P]-ATP (deoxyadenosine 5'-triphosphate tetra-(triethylammonium) salt) were purchased from Amersham (Oakville, Ontario).

Cell culture materials

YT-Indy T-cells and media were a generous gift from Dr. R. C. Bleackley (University of Alberta). Fetal calf serum, non-essential MEM amino acids and geneticin G-418 sulfate were purchased from Gibco/BRL Life Technologies (Burlington, Ontario). Formaldehyde was obtained from Sigma. Vinol 205S was obtained from St. Lawrence Chemical (Toronto, Ontario).

Other

The Matchmaker two-hybrid system was purchased from Clonetech Laboratories. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) and o-nitrophenyl β-D-galactopyranoside (ONPG) was from Sigma. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was from Boehringer Mannheim. Plasmid p5A5 containing the cDNA of human protein disulfide isomerase (Genbank accession no. J02783) was from ATCC (batch 89-06). The cDNA encoding full-length mouse perforin, monoclonal perforin antibodies and purified mouse CTL granules were generous gifts from Dr. R. C. Bleackley (University of Alberta). Rabbit anti-calreticulin N1 antibodies raised against the first 20 amino acids of the human calreticulin sequence were a generous gift from Dr. K.H. Krause (University of Geneva). Anti-SERCA2 IID8 antibody was a generous gift from Dr. K. Campbell (University of Iowa). Rabbit anti-ERp72 was a generous gift from Dr. M. Green (St. Louis University). All chemicals used were of the highest grade available.

METHODS

Synthesis of oligonucleotides and DNA synthesis

All oligonucleotides generated for this work were synthesized by the DNA Synthesis Core Facility in the Department of Biochemistry at the University of Alberta. Synthesis reactions were carried out using an Applied Biosystems 392 DNA/RNA synthesizer.

DNA sequencing was carried out in the same facility using an automated dideoxynucleotide termination method. Templates were prepared using either a modified alkaline lysis procedure recommended by Applied Biosystems Inc. or by a Qiagen column purification kit following the manufacturer's protocol.

Preparation of plasmid DNA

Restriction endonuclease digests, alkaline phosphatase treatment and ligation procedures involved in vector preparation and subsequent cloning procedures were carried out according to accepted published protocols (Sambrook *et al.*, 1989; Ausubel *et al.*, 1988). DNA fragments generated for use in the construction of plasmids for this study were isolated from low melting point agarose gel slices by glass milk powder elution method (Davis *et al.*, 1986). Ligation mixtures were diluted 1:4, transformed into DH5α and plated onto LB agar plates containing 100 μg/mL ampicillin. Individual colonies were selected and grown overnight in LB broth supplemented with ampicillin as before and DNA was isolated using the alkaline lysis method. The resulting DNA was analyzed by restriction endonuclease digest. When required positive cDNA clones were analyzed by DNA sequencing.

PCR reactions were carried out in 100 μl reactions in 10 mM KCL, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.2 supplemented with 200 μM of dNTP, 0.8 μM of the appropriate primers and 20 ng of the appropriate template. The PCR products were concentrated and purified using 30,000 MW cut-off spin filters followed by two washes with ddH_2O.

DNA for transformation into yeast was prepared using the alkaline lysis method. For transfection procedures into mammalian cells, Qiagen column purification kits were used to obtain high purity covalently closed circular (CCC) plasmid DNA. The resulting DNA was precipitated at -70°C for 20 minutes with 2 volumes of 70% ethanol and 1/10 volume of 3 M sodium acetate pH 5.6. The DNA was pelleted at 10,000 *x g* for 20 minutes and washed twice with 70% ethanol. The pellet was dried under sterile conditions and brought up in an appropriate volume of sterile ddH_2O. Quantitation of purified DNA was performed by absorbance spectroscopy.

Production of antibodies

The polyclonal rabbit anti-calreticulin antibodies were raised in New Zealand White rabbits. The antibodies were produced against purified full length mature calreticulin generated in the *Pichia* expression system. The rabbits were injected subcutaneously with 1 mg of the purified protein emulsified in Freund's complete adjuvant. The immunizations were repeated twice using 1 mg of the purified protein emulsified in Freund's incomplete adjuvant at 2 week intervals.

The polyclonal rabbit anti-CRT283 antibodies were also raised in New Zealand White rabbits. The antibodies were produced against a synthetic peptide representing the last six amino acids (NH_2-QAKDEL-COOH) of mature calreticulin. This peptide was synthesized on a peptide synthesizer (Model 430A; Applied Biosystems Inc., Foster City, CA) and coupled to either keyhole limpet hemocyanin or BSA as a carrier protein. The peptide was synthesized with an N-terminal ornithine residue to serve as a spacer between the carrier protein and peptide. Peptide synthesis and purification were carried out by the Alberta Peptide Institute (University of Alberta). The rabbits were injected subcutaneously with 1 mg of the KLH coupled peptide emulsified in Freund's complete adjuvant. At 2 and 4 weeks after the initial injection the immunizations were repeated using 1 mg of the KLH coupled peptide emulsified in Freund's incomplete adjuvant. The resulting antibodies were purified by affinity chromatography. BSA coupled peptide was coupled to activated CNBr Sepharose 4B (Pharmacia) by the manufacturer's protocol. 2 mg of the BSA coupled peptide was rotated with 1 mL of the activated CNBr Sepharose 4B in coupling buffer (500 mM NaCl, 100 mM $NaHCO_3$, pH 8.3) for 16-18 hours at 4°C. Excess unbound peptide was removed by several washes with the coupling buffer. The remaining active groups on the CNBr sepharose 4B column were blocked by running 1 M ethanolamine followed by 100 mM $NaHCO_3$, pH 9.0, through the column. The column was then washed three times with 500 mM NaCl, 100 mM NaAc, pH 4.0 followed by one wash with 500 mM NaCl, 100 mM NaAc, pH 8.0. The CRT283 serum was loaded onto the column and the specific antibodies were eluted with ImmunoPure Gentle Ag/Ab elution buffer (Pierce).

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-PAGE was performed according to Laemmli (1970). Unless otherwise stated 10% polyacrylamide gels were used. The low molecular weight protein markers (BioRad) used were: phorphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). For detection of separated proteins the gels were either stained with Coomassie blue or electrophoretically transferred to nitrocellulose membrane (Towbin *et al.*, 1979) using a semi-dry transfer apparatus.

After transfer, nitrocellulose membranes were stained with Ponceau S to verify that the protein transfer had occurred. The membranes were then blocked in 5% milk powder dissolved in PBS for 1 hour at room temperature with shaking. The blocked membranes were then incubated with primary antibody in 1% milk powder in PBS for 1 to 2 hours. Primary antibodies used were diluted as follows: goat anti-calreticulin (serum), 1:300; monoclonal anti-perforin, 1:100; affinity purified rabbit anti-CRT283, 1:100; anti-SERCA2 IID8, 1:500; anti-BIP, 1:100; anti-ERp72, 1:300; human anti-laminin autoantibodies, 1:200. After incubation in primary antibody the membranes underwent two 10 minute washes in 0.05% Tween-20 in PBS followed by one 10 minute wash in PBS alone. The membranes were then incubated in the appropriate horseradish peroxidase conjugated secondary antibody diluted 1:10,000 in 1% milk powder in PBS for 1 hour. The wash steps were then repeated. Detection of the bound antibodies was by the chemiluminescent ECL detection system (Amersham).

Purification of canine pancreatic calreticulin

Pancreatic calreticulin was purified from fresh dog pancreas by selective ammonium sulfate precipitation according to Baksh *et al.* (1992) and is briefly described here.

Approximately 4 pancreases were homogenized in 4 mL/g tissue of buffer containing 100 mM KH_2PO_4, pH 7.1, 1 mM EDTA and SL inhibitors with 2.66 M $(NH_4)_2SO_4$ (55% saturation) in a blender for 1 minute. The homogenate was then centrifuged at 12,000 *x g* for 30 minutes in a GSA Sorval rotor. The supernatant was filtered through gauze and saved. The pellet was resuspended in half the original volume of the same buffer and rehomogenized. The homogenate was centrifuged again as before. The resulting supernatant was filtered and combined with the first supernatant. The pellet at this point was discarded. 200 g of $(NH_4)_2SO_4$ was added per liter of supernatant with stirring to achieve 85% saturation. This was left to stir at 4°C for 30 minutes. The pH was adjusted to 4.4 by adding phosphoric acid dropwise. The resulting suspension was stirred for 4 hours at 4°C and then centrifuged at 12,000 *x g* for 30 minutes using the same rotor as before. The supernatant was discarded and the pellet was dissolved in 100 mM KH_2PO_4 pH 7.1 and 1 mM EGTA. This was then dialyzed twice for 24 hours each time against 100 mM KH_2PO_4 pH 7.1, 1 mM EGTA and 50 mM NaCl. The suspension was centrifuged at 12,000 *x g* for 30 minutes as before and the supernatant was applied directly to a 60 mL DEAE-Sephadex A-50 column (3 cm x 12 cm; flow rate ~0.6 mL/min; capacity 200 mg) that had been equilibrated with 100 mM KH_2PO_4 pH 7.1, 1 mM EGTA and 50 mM NaCl. The column was washed with 5 volumes of the same buffer. A linear gradient of 50 mM to 750 mM NaCl (~0.83 mM NaCl/minute) in the phosphate buffer was applied to the column and 7 mL fractions collected. Every second fraction was analyzed by 10% SDS-PAGE. Fractions containing calreticulin were pooled and dialyzed for 16 hours against 10 mM KH_2PO_4 pH 7.0. The dialyzed calreticulin was loaded onto a 50 mL hydroxylapatite column (2 cm x 15 cm; flow rate ~0.5 mL/min; capacity 20 mg) and further purified according to MacLennan (1974). The column was washed with three column volumes of 10 mM KH_2PO_4 pH7.0 and the remaining calreticulin bound to the column was eluted with a linear gradient of 10 mM to 1 M KH_2PO_4 pH 7.0 (~1.25 mM KH_2PO_4/minute). Five mL fractions were collected and analyzed by SDS-PAGE. Pure fractions were pooled, concentrated using an Amicon concentrator with a YM-30 membrane and verified by western blotting with anti-calreticulin antibodies. The protein was quantitated and stored in aliquots at -70°C.

Expression and purification of GST fusion proteins

Expression and purification of GST-calreticulin and GST-calreticulin domains was performed by M. Dabrowska. The GST fusion system for the production of a protein of interest in bacteria fused to GST was well established in our lab (Baksh and Michalak, 1991; Baksh *et al.*, 1992). The full length, mature form of calreticulin as well as the N-, P- and C-domains of calreticulin were previously cloned by Kim Burns into the pGEX-3X expression vector to form pGEX-CRT, pGEX-N, pGEX-P and pGEX-C, respectively. These constructs were then transformed and expressed in the protease-deficient *E. coli* strain, BNN103. The resulting fusion proteins had the GST protein fused to the amino terminus of the respective full-length protein or domain being expressed.

The DNA binding domain of the glucocorticoid receptor (GR) was also cloned into the pGEX-3X expression vector by Kim Burns. The cDNA encoding amino acid residues #420-#506 was generated by PCR and cloned into the *BamHI* and *EcoRI* sites of the pGEX-3X plasmid to create pGEX-GR. The following expression and purification protocol applies to the production of GST alone and all of the GST-fusion proteins.

Transformed BNN103 cells were grown in LB broth containing 100 µg/mL of ampicillin to mid-log phase (A_{600}=0.6-1.0). To induce production of the fusion protein, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to the growing culture. The cells were allowed to grow for an additional 4 hours at which point they were harvested by centrifugation at 3,500 *x g* for 15 minutes at 4°C. The resulting pellet was resuspended in 1.0% Triton X-100 in PBS and lysed using French Press set at 1,000 p.s.i.. Lysates were then centrifuged for 10 minutes at 10,000 *x g* to remove cellular debris and insoluble matter.

The supernatants were applied to a 40 mL glutathione-sepharose 4B column (2 cm x 12 cm; flow rate 1 mL/minute; capacity 7.5 mg/ml of beads) equilibrated with 0.1% Triton X-100 in PBS. The column was washed with 5 column volumes of the Triton/PBS buffer. Bound fusion proteins were eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0. Approximately 2.5 mL fractions were collected, analyzed by SDS-PAGE and Western blotting.

In some cases where calreticulin was desired on its own the GST moiety was removed from the fusion protein via factor Xa cleavage resulting in full length, mature, calreticulin referred to as recombinant calreticulin. This was achieved by incubating GST-CRT with factor Xa diluted 1:500 (w/v) in 100 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris-HCl, pH 8.0 for 16 hours at room temperature. The reaction was stopped by the addition of 2.5 mM PMSF (final concentration). This digested sample was reloaded onto the GST affinity column and the resulting recombinant calreticulin in the flow-through was collected, concentrated and dialyzed against 50 mM NaCl, 100 mM KH_2PO_4, 1 mM EDTA, pH 7.1. This was directly loaded onto a Mono Q 5/5 anion exchange FPLC column (flow rate 1 mL/min) equilibrated in the same buffer. After washing the column extensively a linear gradient of 0 - 750 mM NaCl in 30 minutes (25 mM B/min) was run. Fractions were analyzed by SDS-PAGE and western blotting. The recombinant calreticulin eluted at approximately 300 mM NaCl. Fractions containing pure recombinant calreticulin were pooled, concentrated and stored at -70°C. The protein yield was quantitated using the DC protein concentration kit

For the purification of GST-GR the fractions containing the GST-GR were eluted from the GST affinity column followed by dialysis against 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 40 mM Tris-HCl, pH 7.2. This was then loaded onto a calreticulin affinity column equilibrated in the same buffer. The column was washed with 5 mM EDTA, 100 mM NaCl and 40 mM Tris-HCl, pH 7.2 and the GST-GR was eluted with a high salt buffer of 20 mM EDTA, 750 mM NaCl and 40 mM Tris-HCl, pH 7.2. The purified GST-GR was concentrated, quantitated and then used in the DNA mobility shift assay.

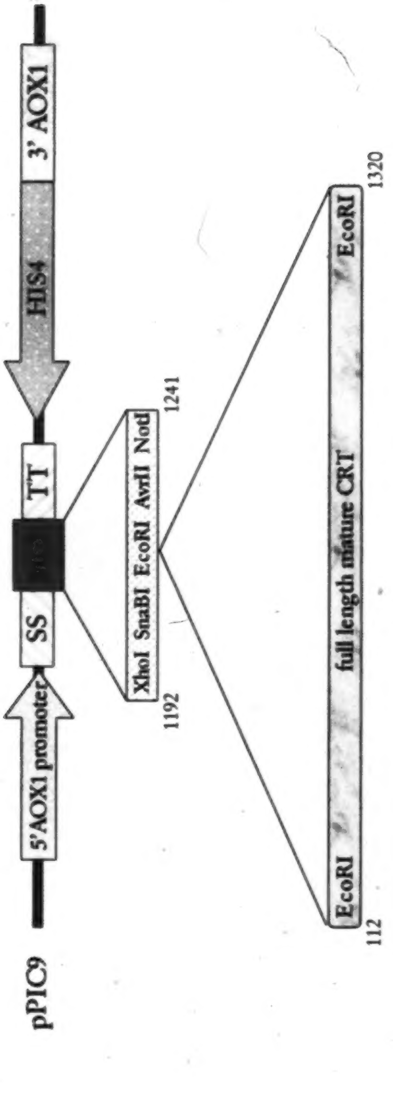
Calreticulin expression and purification from the *Pichia* expression system

The cDNA of full length rabbit calreticulin, without a signal sequence, was obtained by restriction endonuclease digestion of pGB-CRT (Baksh *et al.*, 1995) with *EcoRI* and gel purification of the resulting DNA fragment. The *Pichia* expression plasmid, pPIC-9, was digested with *EcoRI* endonuclease, treated with calf intestinal alkaline phosphatase and gel purified. The CRT DNA fragment was then ligated into the prepared pPIC-9 plasmid to generate pPIC-CRT (Fig. 2-1). The presence of calreticulin cDNA and its orientation in the plasmid was determined by endonuclease digestion. All nucleotide sequences were confirmed by DNA sequencing.

pPIC-CRT was transformed into the KM71 strain of *Pichia* by the protocol outlined by the manufacturer. Briefly, an initial culture of KM71 was grown overnight in Yeast Extract Peptone Dextrose media (YPD) at 30°C with vigorous shaking. This confluent culture was then used to inoculate 200 mls of YPD. This was grown overnight under the same conditions until an $O.D._{600}$ of 0.240 was reached. Cells were then harvested by centrifugation at 1500 *x g* for 10 min. Cells were washed once with sterile water, once with 1 M sorbitol, 25 mM EDTA and 5 mM DTT and once with 1 M sorbitol centrifuging as before between each wash. Cells were then resuspended in 20 mls of 1 M sorbitol, 1 mM

Figure 2.1: Structure of pPIC-CRT

This is a schematic representation of the pPIC-CRT cDNA clone constructed for the expression of rabbit CRT in the *Pichia* protein expression system. The cDNA encoding rabbit CRT was cloned into the *EcoRI* restriction site of the multiple cloning site (MCS) on the pPIC9 vector. The cDNA was cloned downstream of the 5' alcohol oxidase 1, AOX1, promoter and α factor signal sequence (SS) and upstream of a transcription termination signal (TT). The pPIC9 vector also contains a 5' and 3' AOX1 specific sequence to allow for incorporation of the CRT cDNA into the yeast genome at the AOX1 site. Nutritional selection of the transformants is achieved by the inclusion of the HIS4 gene as shown on the diagram.


pPIC9
5'AOX1 promoter
SS
TT
HIS4
3' AOX1
XhoI SnaBI EcoRI AvrII NotI
1192
1241
EcoRI
full length mature CRT
EcoRI
112
1320

EDTA, 10 mM sodium citrate pH 5.8. Half of the cells were then incubated with 3 units/ml of lyticase for 22 minutes resulting in approximately 70% of the cells as spheroplasts. Spheroplasts were then harvested by centrifugation at 750 *x g* for 10 minutes at room temperature. Spheroplasts were washed once with 1 M sorbitol and once with 1 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$ centrifuging between each wash. Prepared spheroplasts were then resuspended in 0.6 ml of the last wash buffer. Ten μg of linearized plasmid was incubated with 100 μl of the spheroplast preparation for 10 minutes at room temperature. Twenty % PEG, 10 mM Tris-HCl pH7.5, and 20 mM $CaCl_2$ was added and incubated for an additional 10 minutes. This mixture was centrifuged at 750 *x g* for 10 minutes. The pellet was resuspended in 100 μl of 1 M sorbitol, 10 mM $CaCl_2$ in diluted YPD and incubated for an additional 10 minutes. Eight hundred and fifty μl of 1 M sorbitol was then added. The transformation mixture was then diluted 1:330 with regeneration dextrose, without histidine, molten agar and plated in triplicate on the same agar plates and grown for seven days at 30°C. Transformants were then isolated and grown on minimal dextrose agar plates. DNA was isolated from individual transformants and analyzed by PCR using appropriate primers supplied by the manufacturer. Transformants having incorporated the calreticulin coding region from the pPIC-CRT plasmid were then tested for protein expression. Transformants were grown in Buffered Minimal Glycerol media for 18 hours at 30°C. Cells were harvest by centrifugation at 3000 *x g* for 5 minutes at room temperature and resuspended in Buffered Minimal media containing 0.5% methanol to induce protein production. Protein expression and secretion into the media was analyzed at 24, 48, and 72 hours by SDS-PAGE. 100% methanol was added every 24 hours to a final concentration of 0.5% to maintain induction. Optimum expression and secretion into the media was found after 72 hours. The media obtained after 72 hours was concentrated approximately 8 fold using an Amicon filter with a 30,000 MW cut-off. This was then loaded onto a DEAE-sephadex column (3 cm x 12 cm; flow rate 0.6 mL/minute; capacity 200 mg) and washed with 3 column volumes of low salt buffer; 50 mM NaCl, 100 mM KH_2PO_4, 1 mM EDTA, pH 7.1. The protein was eluted using a linear high salt gradient prepared in the low salt buffer and run at a rate of ~0.9 mM KH_2PO_4/minute to a final concentration of 800 mM NaCl. Five mL fractions were collected and analyzed by SDS-PAGE. The pure fractions were pooled and concentrated as before. The buffer was exchanged by doing several washes in the Amicon concentrator using buffer A; 20 mM NaH_2PO_4, Na_2HPO_4 pH 7. The resulting protein fraction was further purified using a 6 mL prepacked Resource Q FPLC column (Pharmacia). This anion exchange column is faster and easier to run due to its small size yet has a much higher capacity than the Mono Q anion exchange column. Approximately 100 mg of protein was loaded onto the column and washed with three column volumes of buffer A. The protein was eluted using a high salt gradient of buffer B (780 mM NaCl in buffer A) at ~7 mM B/minute holding the gradient at the elution of each protein peak. 1 mL fractions were collected and subsequently analyzed using 10% SDS-PAGE. Pure fractions were pooled and concentrated as before and quantitated using either the Lowry *et al.* (1951) or the Bradford (1976) method. Identity of the pure fractions was verified by Western blot.

DNA mobility shift assay

The GRE used in this assay was synthesized as two complimentary oligodeoxynucleotides: 5'-TCC TTG TTT TAA GAA CAG TTA TCG ATT ATA AAC-3' and 5'-GTT TAT AAT CGA TAA CGT TTC TTA AAA CAA GGA. Equimolar amounts of the two oligodeoxynucleotides were mixed together and boiled for 5 minutes. The mixture was then cooled slowly to allow the oligodeoxynucleotides to anneal. The resulting double stranded GRE was then radioactively labeled with $\gamma[^{32}P]$-ATP and T4 kinase (Gibco) for 30 minutes at 37°C in the kinase buffer provided by the manufacturer. The reaction was stopped with 0.5 M EDTA. The mixture was then loaded onto a 1 mL G-50 Sephadex column prepared in a 1 cc syringe. The flow through contained the radioactively labeled GRE and was used directly in the mobility shift assay. All reactions were carried out in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM NaCl, 0.05% BSA, 4 mM DTT, 10 % glycerol and 2 μg poly-dI:dC. Dog pancreatic calreticulin, GST-GR and buffer were mixed together and allowed to incubate for 15 minutes at room temperature. The labeled GRE was then added and the reaction was incubated for an additional 30 minutes at room temperature. In the case where the GRE and the GR were allowed to bind before the addition of calreticulin, the labeled GRE, GST-GR and buffer were incubated together for 15 minutes at room temperature, calreticulin was added and allowed to incubate for the additional 30 minutes. The reactions were then loaded onto a 5% polyacrylamide gel in 22 mM Tris-HCl, pH 7.5, 22 mM boric acid and 0.5 mM EDTA. The gel was run at 100 volts for 3.5 hours, dried and exposed to X-ray film for 24-48 hours.

Isolation, fractionation and immunostaining of nuclei

Nuclei were isolated with the assistance of Jody Groenendyk by the procedure of Blobel and Potter (1966). Fresh rat liver was homogenized in 2 volumes of 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5. The homogenate was placed on a gradient of 1.62 M and 2.3 M sucrose. The step gradient was centrifuged at 124,000 *x g* for 30 minutes using a Beckman SW 50.1 rotor. The pellet consisted of pure nuclei and was washed by resuspension in 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5 and centrifuging at 13, 000 *x g* for 12 minutes. The resulting pellet contained pure, intact nuclei that were then used for staining or fractionation.

The pure nuclei were extracted with 5% Triton X-100 in 25 mM KCl, 5 mM MgCl$_2$, and 50 mM Tris-HCl, pH 7.5. This was then centrifuged for 5 minutes at 800 *x g* to produce an insoluble fraction containing the nuclei with its inner nuclear membrane intact and a soluble fraction containing the stripped off, outer nuclear membrane. To prevent contamination of the stripped nuclei with the solubilized outer membrane, the insoluble pellet was washed in 25 mM KCl, 5 mM MgCl$_2$, and 50 mM Tris-HCl, pH 7.5, centrifuged at 13, 000 *x g* for 10 minutes and resuspended in the same buffer. The quality of the nuclei was monitored by electron microscopy (Blobel and Potter, 1966)

The pure intact nuclei were allowed to attach to coverslips coated with 1 mg/mL of poly-L-lysine. The nuclei were fixed with 3.7% formaldehyde in PBS, permeablized for 3 minutes with 0.1% Triton X-100, 100 mM PIPES, 1 mM EGTA, 4% polyethylene glycol 8000, pH 6.9 and washed for 10 minutes with PBS. The nuclei were then incubated with either goat or rabbit anti-calreticulin (Opas *et al.*, 1991; Nauseef *et al.*, 1995) at a dilution of 1:50 in 1% milk powder in PBS for 30 minutes. This was followed by incubation with the either FITC-conjugated donkey anti-goat or Texas Red-conjugated donkey anti-rabbit secondary antibodies (1:30 dilution) in 1% milk powder in PBS. After three 5 minute washes the coverslips were mounted in Vinol 205S containing 0.25% 1, 4-diazabicyclo-(2, 2, 2)-octane and 0.002% *p*-phenylenediamine. Confocal microscopy was then performed by Dr. M. Opas (University of Toronto).

Construction of expression vectors for the Matchmaker Two Hybrid system

The expression vectors pGBT9 and pGAD424, provided by the manufacturer were used to generate clones encoding for either the GAL4 DNA binding domain or the GAL4 activating domain, respectively, fused to proteins of interest.

Full length, mature calreticulin and the various domains (Fig. 2-2) were all generated by PCR for cloning into the pGBT9 vector to generate pGB-CRT (Fig. 2-3), pGB-N, pGB-N1 (Fig. 2-4), pGB-P and pGB-P1 (Fig. 2-5) (Baksh *et al.*, 1995). Each calreticulin PCR product was generated to include a 5' *EcoRI* site and a 3' *SalI* site. Primers designed and used for the two hybrid system are listed in Table 2-1. The PCR reactions were carried out in 100 µL reactions using 20 ng of template, 0.8 µM of the appropriate primers, and 200 µM of each dNTP in 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$ and 0.1% Triton X-100. Each reaction was overlaid with mineral oil and heated to 94°C for 3 minutes before initiation of the reaction by the addition of 1 unit of Vent polymerase. For each of the various domains the amplification of the DNA was carried out for 25 cycles consisting of 1 minute at 94°C, 1 minute at 57°C and 2 minutes at 72°C. This was followed by one 10 minute extension cycle at 72°C. To generate the cDNA encoding full-length calreticulin the same PCR reaction was carried out except the annealing temperature was 50°C for 1 minute. PCR products were purified and concentrated using a 30,000 MW cut-off spin filter. Each PCR reaction was placed in a spin filter with 300 µL of sterile ddH_2O and centrifuged at 5,000 rpm for 20 minutes. The filtrate was discarded and the PCR product remaining on the filter was saved. The PCR product was washed twice more by the addition of 400 µL of sterile ddH_2O and centrifuging as before. The PCR products were then digested with *EcoRI* and *SalI* restriction enzymes and gel purified. The pGBT9 vector was digested with the *EcoRI* and *SalI* restriction enzymes and CIP treated for use in the ligation reactions.

Figure 2.2: Putative calreticulin domains

This is a schematic diagram of the putative domains of calreticulin as predicted from the amino acid sequence as shown in Fig. 1-3. Included is a representation of the different domains generated in cDNA clones constructed for use in the yeast two-hybrid system below the model of the protein. As shown, CRT contains an N-terminal signal sequence and a C-terminal ER retention/retrieval sequence. The general location of the two sets of three conserved amino acid repeats are shown by the light and dark grey rectangles. Calcium binding is depicted by the dark circles showing the high capacity binding ability of the C-domain and the low capacity of the P-domain. The putative N-linked glycosylation site as residue 327 is marked by an asterisk (*).

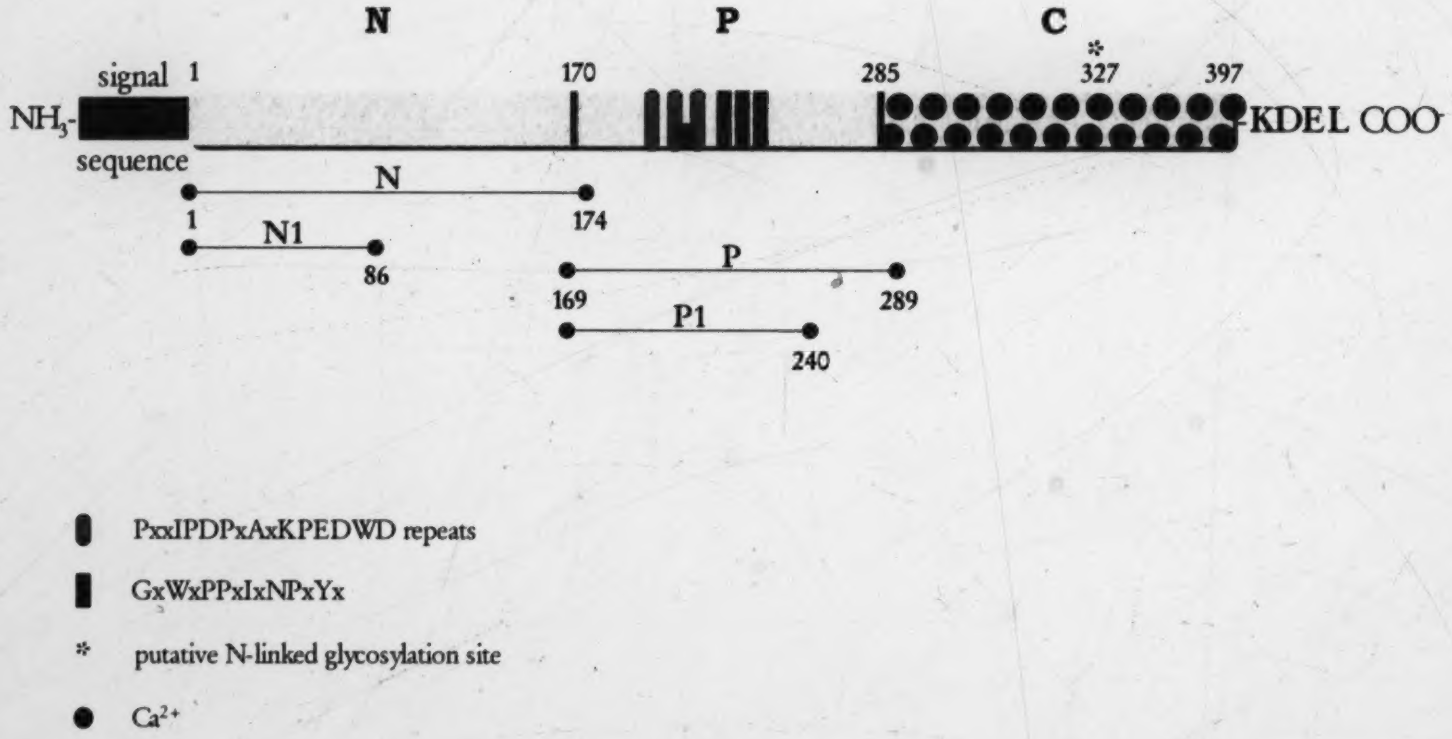
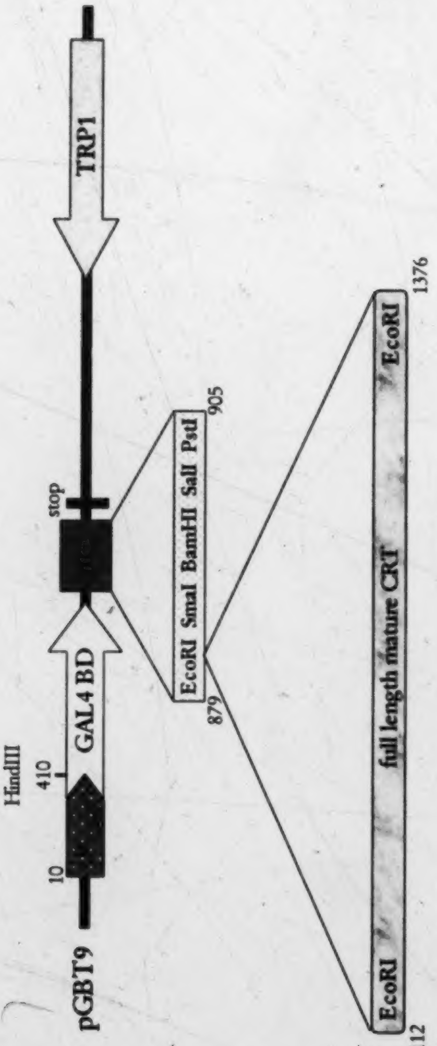

N
P
C
*
signal
sequence
NH3-
1
170
285
327
397
KDEL COO-
N
1
174
N1
86
P
169
289
P1
240
PxxIPDPxAxKPEDWD repeats
GxWxPPxIxNPxYx
putative N-linked glycosylation site
Ca2+

Figure 2-3: Structure of pGB-CRT

This is a schematic representation of the pGB-CRT cDNA clone constructed for use in the yeast two-hybrid system. The cDNA of full length rabbit CRT containing nucleotides 112-1376 was cloned into the *EcoRI* restriction site of the multiple cloning site (MCS) on the pGBT9 vector. The CRT cDNA was cloned downstream of the cDNA encoding the GAL4 DNA binding domain (GAL4 BD) under the control of the ADH1 promoter (P_{ADH1}). Nutritional selection of the transformants is achieved by the inclusion of the TRP1 gene as shown on the diagram.


pGBT9
10
HindIII
410
GAL4 BD
stop
TRP1
EcoRI SmaI BamHI SalI PstI
879
905
EcoRI
full length mature CRT
EcoRI
112
1376

pcDx-CRT containing the cDNA for rabbit skeletal muscle calreticulin (Fliegel *et al.*, 1989) was linearized by digestion with restriction enzyme, *SmaI*, and used as the template DNA in the PCR reactions performed to generate the calreticulin inserts. The CA6 primer was used as the 5' (forward) primer for full length, mature calreticulin as well as for the N and N1 domains. The nucleotide sequence of primer CA6 contained the *EcoRI* restriction site and corresponded to nucleotides 112-131 of the cDNA encoding the first 6 amino acids of mature calreticulin. For full length calreticulin, the 3' (reverse) primer, MMI11, contained the restriction site *EcoRI* and corresponded to the nucleotides 1322-1376. The 3' (reverse) CA11 primer was used with to generate the N1 domain. This primer contained the *SalI* restriction site and corresponded to nucleotides 352-372 encoding for amino acids 80-86. For the cDNA encoding the N domain the 3' (reverse) primer CA13 was used. CA13 contained the *SalI* restriction site and corresponded to nucleotide 613-633 of calreticulin encoding for amino acids residues 168-174.

The 5' (forward) primer CA14 was used to generate both the P1 and P domains and contained the *EcoRI* site and corresponded to nucleotides 616-636 encoding for amino acids residues 169-175. For the P1 domains the CA15 primer was used as the 3' (reverse) primer containing the *SalI* restriction site and corresponded to nucleotides 811-831 of mature calreticulin encoding for amino acids 234-240. The 3'(reverse) primer used to generate the P domain was CA16 containing the *SalI* site and corresponding to nucleotides 958-979, which encodes for amino acids 283-289.

To study the interactions between calreticulin and the GR the GR was cloned downstream from the GAL4 activating domain resulting in the plasmid pGAD-GR (Fig. 2-6). The DNA binding domain of the GR, consisting of amino acids 420-506, was generated

Figure 2-4: Structure of plasmids pGB-N and pGB-N1

This is a schematic representation of the pGB-N and pGB-N1 cDNA clones constructed for use in the yeast two-hybrid system. The cDNA containing nucleotides 112-633 (N) or nucleotides 112-372 (N1) of rabbit CRT was cloned into the *EcoRI* and *SalI* restriction sites of the multiple cloning site (MCS) on the pGBT9 vector. The N-domain cDNAs were cloned downstream of the cDNA encoding the GAL4 DNA binding domain (GAL4 BD) under the control of the ADH1 promoter (P_{ADH1}) followed by a stop codon (stop). Nutritional selection of the transformants is achieved by the inclusion of the TRP1 gene as shown on the diagram.

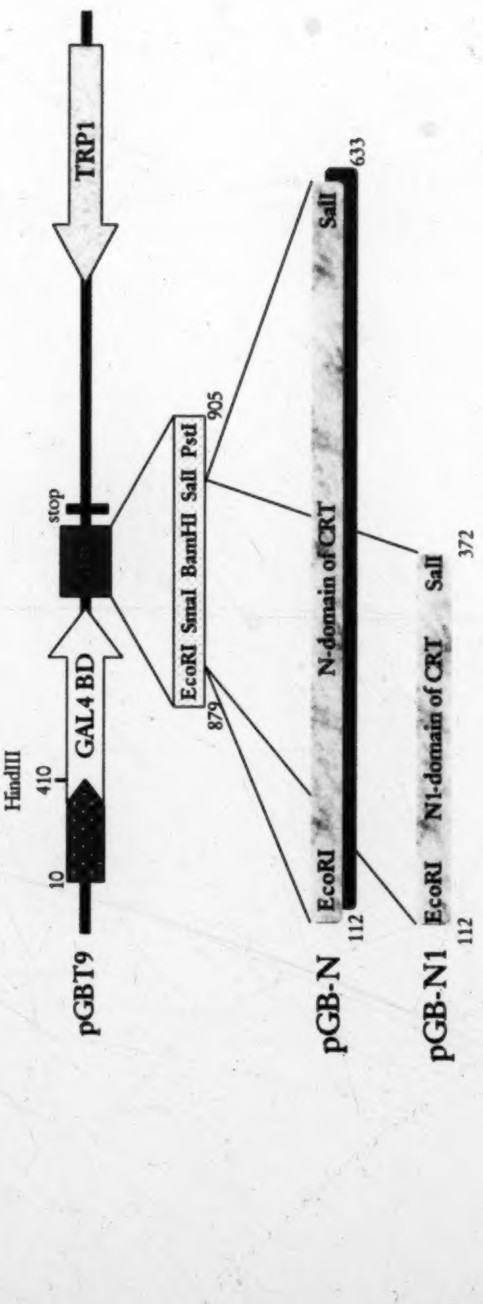

pGBT9
10
HindIII
410
GAL4 BD
stop
TRP1
EcoRI SmaI BamHI SalI PstI
879
905
pGB-N
EcoRI
N-domain of CRT
SalI
112
633
pGB-N1
EcoRI
N1-domain of CRT
SalI
112
372

Figure 2-5: Structure of plasmids pGB-P and pGB-P1

This is a schematic representation of the pGB-P and pGB-P1 cDNA clones constructed for use in the yeast two-hybrid system. The cDNA containing nucleotides 616-978 (P) or nucleotides 616-831 (P1) of rabbit CRT was cloned into the *EcoRI* and *SalI* restriction sites of the multiple cloning site (MCS) on the pGBT9 vector. The P-domain cDNAs were cloned downstream of the cDNA encoding the GAL4 DNA binding domain (GAL4 BD) under the control of the ADH1 promoter (P_{ADH1}) followed by a stop codon (stop). Nutritional selection of the transformants is achieved by the inclusion of the TRP1 gene as shown on the diagram.

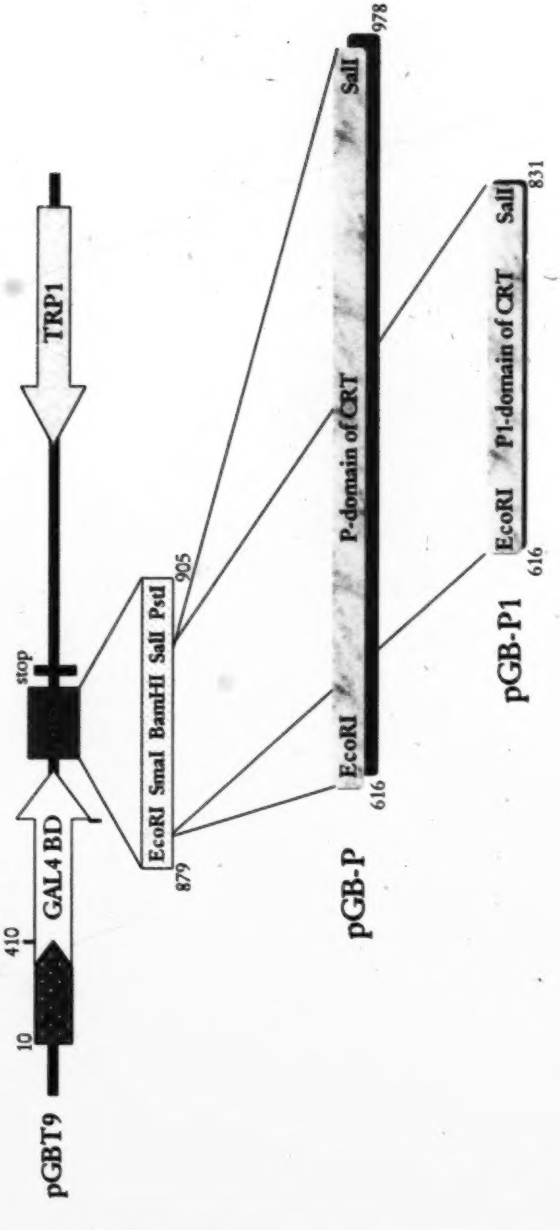

pGBT9
10
410
HindIII
GAL4 BD
stop
TRP1
EcoRI SmaI BamHI SalI PstI
879
905
pGB-P
EcoRI
P-domain of CRT
SalI
616
978
pGB-P1
EcoRI
P1-domain of CRT
SalI
616
831

Table 2-1. Nucleotide sequences of synthetic oligonucleotide primers

Primer Name	Nucleotide sequence	Restriction Site	Nucleotide position
CRT primers			
CA6	atatgaattcGAGCCCGTCGTCTACTTCAA	*EcoRI*	112-131
CA11	atatgtcgacGTCAATGTTCTGCTCGTGTTT	*SalI*	352-372
CA13	atatgtcgacCACCTGGCTGTTGTCAATCTT	*SalI*	613-633
CA14	atatgaattcATTGACAACAGCCAGGTGGA	*EcoRI*	616-635
CA15	atatgtcgacCATTTCTTCGTCCCAGTCTTC	*SalI*	811-831
CA16	atatgtcgacATAGATGTTAGCGTCGGGCGA	*SalI*	959-978
MMI11	gggaattcAGAGACATTATTTGGCTCTGCG	*EcoRI*	1322-1376
GR primers			
A	atggatcCTCTGCCTGGTGTGCTCTGATGAA	*BamHI*	1389-1403
B	atggatccTCCTGTAGTGGCCTGCTGAATTCC	*BamHI*	1626-1650
PDI primers			
CA7	atatgaattcGACGCCCCCGAGGA	*EcoRI*	115-135
CA8	atatggatccGGGTCTGGCTTTGCGTA	*BamHI*	1591-1607
Perforin primers			
CA21	atatgaattcATGCCCTGCTACACTGCCAC	*EcoRI*	61-77
CA22	atatggatccTTACCACACAGCCCCACTG	*BamHI*	1647-1665

Table 2-1: Primer sequences are shown in a 5' to 3' orientation. The capitalized bases represent the cDNA sequence encoding the protein to be amplified. Bases in small letters represent additional nucleotides added to create restriction sites (underlined) for ease of cloning.

by PCR as described in Burns *et al.*, (1994). The pOB10 plasmid, which encodes for the β-form of the human GR was linearized and used as the template in the PCR reaction. Primer A, used as the 5' (froward) primer contained a *BamHI* restriction site and corresponded to nucleotides 1389-1403. The 3' (reverse) primer, designated primer B, also contained a *BamHI* site and corresponded to nucleotides 1626-1650. The PCR reaction was carried out as stated above using 20 ng of template, 0.8 μM of the appropriate primers, and 200 μM of each dNTP in 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$ and 0.1% Triton X-100. the reaction was initiated by the addition of 1 unit of Vent polymerase after incubating the reaction for 3 minutes at 94°C. The DNA amplifications were carried out for 25 cycles consisting of 1 minute at 94°C, 1 minute at 57°C and 2 minutes at 72°C. This was followed by one 10 minute extension cycle at 72°C. The PCR product was purified by spin filter, digested with *BamHI* and gel purified. The DNA fragment was then ligated into pGAD424 that had been previously digested with *BamHI*, CIP treated and gel purified. For the studies with PDI, the full length, mature PDI was cloned downstream from the GAL4 activating domain in pGAD424 (Fig. 2-7). The cDNA encoding PDI was generated by PCR amplification of p5A5 vector containing the cDNA of full-length human PDI (Genbank accession no. J02783). The PCR reactions were carried out as stated above for the calreticulin domains in the same buffer. Primer CA7 was used as the 5' (forward) primer containing an *EcoRI* restriction site and corresponded to nucleotides 115-135. The 3' (reverse) primer was CA8 and included a *BamHI* restriction site and corresponded to nucleotides 1591-1607.

Figure 2-6: Structure of pGAD-GR

This is a schematic representation of the pGAD-GR cDNA clone constructed for use in the yeast two-hybrid system. The cDNA of the DNA binding domain of the glucocorticoid receptor (GR) containing nucleotides 1389-1650 was cloned into the *BamHI* restriction site of the multiple cloning site (MCS) on the pGAD424 vector. The GR cDNA was cloned downstream of the cDNA encoding the GAL4 activating domain (GAL4 AD) under the control of the ADH1 promoter (P_{ADH1}) followed by a stop codon (stop). Nutritional selection of the transformants is achieved by the inclusion of the LEU1 gene as shown on the diagram.

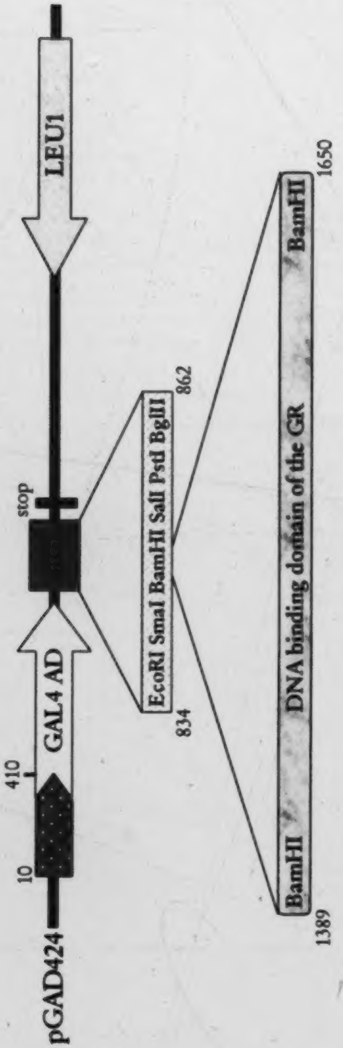
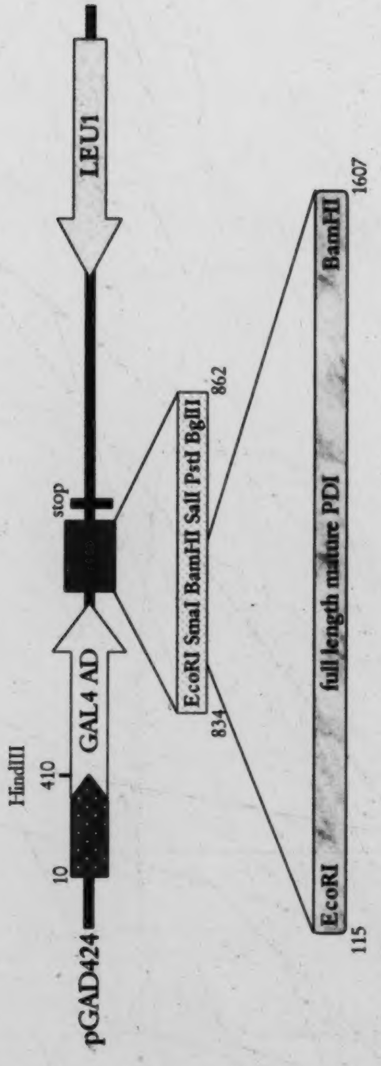

pGAD424
10
HindIII
410
GAL4 AD
stop
LEU1
EcoRI SmaI BamHI SalI PstI BglII
834
862
BamHI
DNA binding domain of the GR
BamHI
1389
1650

Figure 2-7: Structure of pGAD-PDI

This is a schematic representation of the pGAD-PDI cDNA clone constructed for use in the yeast two-hybrid system. The cDNA of full length human protein disulfide isomerase (PDI) containing nucleotides 115-1607 was cloned into the *EcoRI* and *BamHI* restriction sites of the multiple cloning site (MCS) on the pGAD424 vector. The PDI cDNA was cloned downstream of the cDNA encoding the GAL4 activating domain (GAL4 AD) under the control of the ADH1 promoter (P_{ADH1}) followed by a stop codon (stop). Nutritional selection of the transformants is achieved by the inclusion of the LEU1 gene as shown on the diagram.


pGAD424
10
HindIII
410
GAL4 AD
stop
LEU1
EcoRI SmaI BamHI SalI PstI BglII
834
862
EcoRI
full length mature PDI
BamHI
115
1607

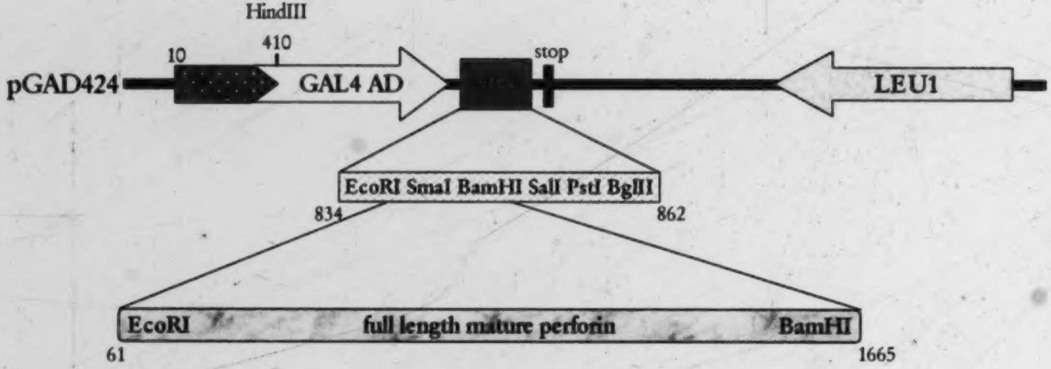
To examine the interaction between calreticulin and perforin in the two hybrid system the cDNA perforin was cloned into the pGAD424 vector to generate pGAD-perf (Fig. 2-8). The cDNA encoding full-length mature perforin was generated by PCR amplification. A cDNA encoding mouse perforin isolated from a mouse T-cell DNA library was used as the template. Primer CA21 was used as the 5' (forward) primer in the reaction. It included an *EcoRI* site and encoded for the first 6 amino acids of mature perforin. Primer CA22 was used as the 3' (reverse) primer containing a *BamHI* restriction site and encoded for the last 5 amino acids of mature perforin. The PCR reaction was carried out under the same buffer conditions and the same concentrations of primers and template as stated for the above reactions. The amplification reaction was carried out for 25 cycles consisting of 1 minute at 94°C, 1 minute at 50°C and 2 minutes at 72°C, followed by one 10 minute extension cycle at 72°C. The PCR product was purified by spin filter, digested with *EcoRI/BamHI* and gel

Matchmaker Two Hybrid system

To examine protein-protein interactions within a cellular environment the Matchmaker two hybrid system (Clonetech) was employed. This yeast two hybrid system (Fields and Song,1989; Chien *et al.*, 1991) is based on the fact that the GAL4 transcription factor can be broken into two domains, a DNA binding domain and a DNA activating domain. When brought back together, in close enough proximity to each other, a functional transcription factor is reconstituted that can then activate a reporter gene activity that is under GAL4 control. In the case of the Matchmaker system, the reporter gene used is the β-galactosidase gene. By cloning cDNAs of proteins of interest into the matchmaker vectors we created the constructs, described in the preceding section, that express our proteins as fusion proteins with either the GAL4 activating or DNA binding domain and target the hybrid proteins to the yeast nucleus. Appropriate constructs are transformed into *Saccharomyces cerevisiae*,

Figure 2-8: Structure of pGAD-perforin

This is a schematic representation of the pGAD-perforin cDNA clone constructed for use in the yeast two-hybrid system. The cDNA of full length mouse perforin containing nucleotides 61-1665 was cloned into the *EcoRI* and *BamHI* restriction sites of the multiple cloning site (MCS) on the pGAD424 vector. The perforin cDNA was cloned downstream of the cDNA encoding the GAL4 activating domain (GAL4 AD) under the control of the ADH1 promoter (P_{ADH1}) followed by a stop codon (stop). Nutritional selection of the transformants is achieved by the inclusion of the LEU1 gene as shown on the diagram.


HindIII
10
410
pGAD424
GAL4 AD
stop
LEU1
EcoRI SmaI BamHI SalI PstI BglII
834
862
EcoRI
full length mature perforin
BamHI
61
1665

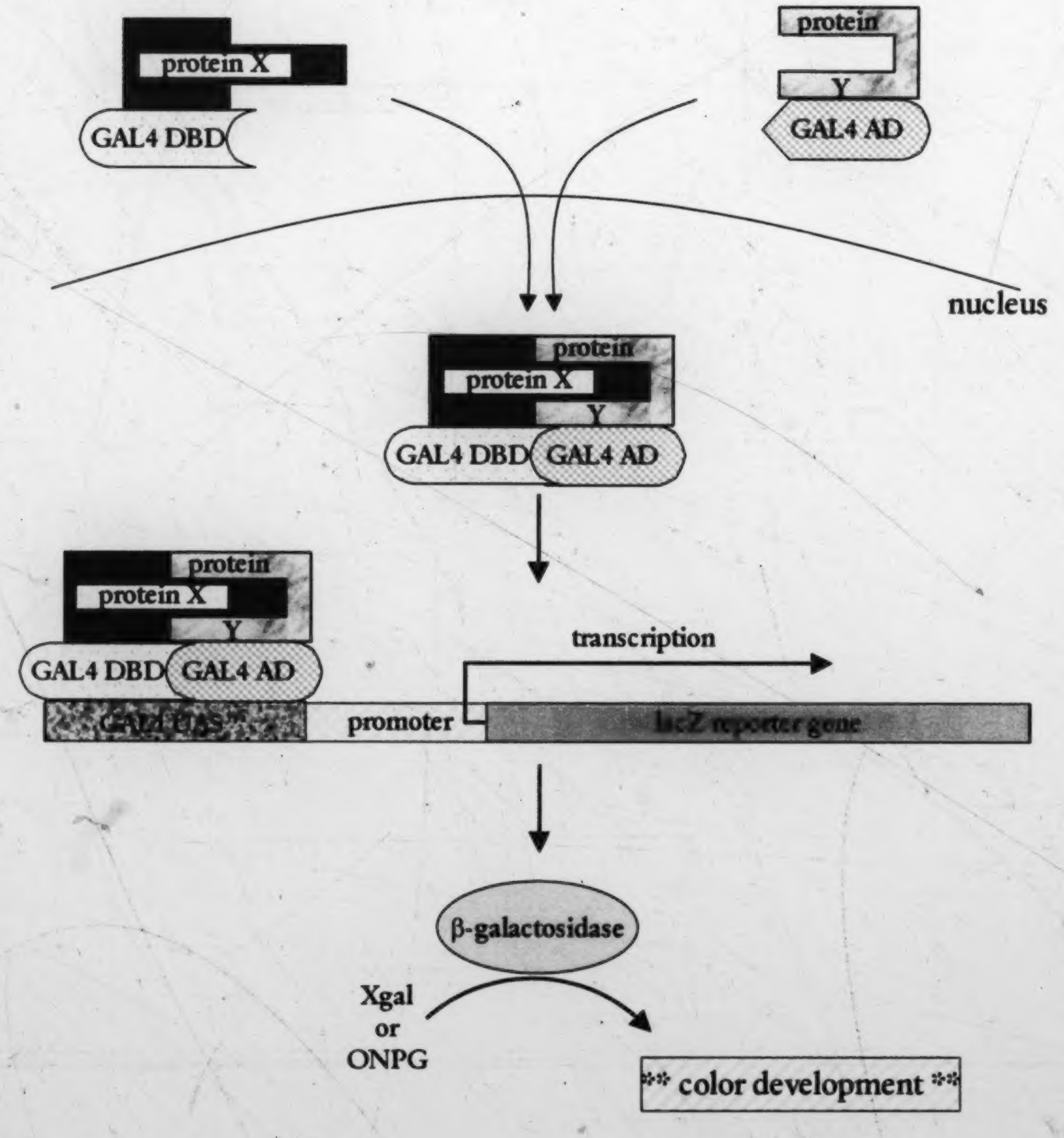
allowed to express and then examined for β-galactosidase activity which reflects whether or not the two proteins examined interact within the yeast nucleus (Fig. 2-9).

The desired constructs were introduced into the SFY526 strain of *S. cerevisiae* by the lithium acetate method (Ito *et al.*, 1983; Schiestl and Gietz, 1989; Hill *et al.*, 1991; Gietz *et al.*, 1992). pGB-CRT, pGB-N1, pGB-N2, pGB-P1 or pGB-P2 were transformed alone or in combination with pGAD424, as a control, or with pGAD-GR, pGAD-PDI or pGAD-perf to examine potential calreticulin-protein interactions. As well, pGAD-GR, pGAD-PDI or pGAD-perf was transformed alone or in combination with pGBT9 as controls.

The appropriate plasmid DNA was prepared in eppendorf tubes with 100 μg of salmon sperm DNA as a carrier. For the introduction of single plasmids, 100 ng of DNA was used per transformation and for double transformations 200 ng of DNA was used of each plasmid per transformation. One hundred μL of competent cells were then added followed by 600 μL of 50% PEG 4000. The reactions were then vortexed to ensure thorough mixing and incubated at 30°C for 30 minutes. Cells were then shocked by the addition of 70 μL of DMSO and incubation at 42°C for 15 minutes. The transformation mixtures were placed on ice for 2 minutes and the cells were pelleted by centrifugation at 10,000 *x g* for 5 seconds. The supernatant was aspirated and the pelleted cells were resuspended in 300 μL of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The cells were then plated on the appropriate synthetic dropout (SD) agar plates lacking either leucine, tryptophan or both amino acids to allow for the selection of transformants. Cells were allowed to grow for 7 days at 30°C. To analyze whether or not the proteins introduced interact, a rapid, colony lift β-galactosidase filter assay was performed.

Figure 2-9: Schematic diagram of the Yeast Two-Hybrid system

This illustration depicts the general principles behind the yeast two-hybrid system. A protein of interest (protein X) is generated fused to the GAL4 DNA binding domain (GAL4 DBD) and a second protein of interest (protein Y) is generated fused to the GAL4 activating domain (GAL4 AD). The fusion proteins are targeted to the yeast nucleus. If the two proteins of interest interact in the yeast nuclear environment then the two domains of the GAL4 transcription factor are brought into close enough proximity to reconstitute a functional transcription factor. This then activates the transcription of a reporter gene (lacZ reporter gene, in this case) under the control of a GAL4 upstream activating sequence (GAL4 UAS). β-galacotsidase is subsequently produced and can be detected by providing the appropriate substrate (Xgal or ONPG) and assaying for color development. The color detected is then a measure of the interaction occurring between the proteins of interest.


protein X
GAL4 DBD
protein
Y
GAL4 AD
nucleus
protein
protein X
Y
GAL4 DBD
GAL4 AD
protein
protein X
Y
GAL4 DBD
GAL4 AD
transcription
GAL1 UAS
promoter
lacZ reporter gene
β-galactosidase
Xgal
or
ONPG
** color development **

For the colony lift assay a sterile Whatman #5 filter paper was presoaked in 2.5 mLs of 330 µg X-gal in 40 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, for every plate of transformants being assayed. Another sterile, dry filter paper was then placed on the plate of transformants and pressed down gently. The yeast cells were permeablized by removing the filter from the agar plate and laying it onto a pool of liquid nitrogen colony side up. The filter was submerged for approximately 30 seconds to allow the cells to freeze completely. The filter was then removed and allowed to thaw before layering it onto the presoaked filter. Blue color development of the individual colonies was monitored for 24 hours.

In the instance where it was desirable to quantitate the interaction between the proteins being examined a liquid culture β-galactosidase assay was performed. For this assay single transformants were inoculated into 5 mL of SD media lacking the appropriate amino acid(s) to maintain selection. The culture was grown overnight at 30°C with shaking at 250 rpm. Two mL of the overnight culture was transferred to 8 ml of YPD media and allowed to grow for an additional 3-5 hours until an $O.D._{600}$ of 0.5-0.8 was reached. The culture was vortexed to thoroughly disperse the culture and the exact $O.D._{600}$ recorded. The remainder of the procedure was performed in triplicate for each transformant being assayed. 1.5 mL of culture was centrifuged at 10,000 *x g* for 30 seconds. The pellets were washed in 40 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, re-centrifuged and resuspended in 300 µL of the same buffer. One hundred µL of the cells were transferred to a fresh tube and permeablized by placing in liquid nitrogen for 30 seconds and then transferring to a 37°C water bath for 30 seconds. The cells were then diluted with 700 µL of

0.275 β-mercaptoethanol in 40 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0. 160 μL of ONPG dissolved in the phosphate buffer (4 mg/ml) was added. The reactions were incubated at 30°C and color development monitored for 24 hours. The cells were then centrifuged at 10,000 *x g* for 10 minutes to remove all cellular debris. The $O.D._{420}$ of the supernatant was measured and the β-galactosidase units were calculated according to the manufacturer as follows:

$$\beta\text{-galactosidase units} = 1000 \times O.D._{420}/(t \times V \times O.D._{600})$$

where t = elapsed time in minutes

V = 0.1 mL x concentration factor

OD_{600} = A_{600} recorded of the starting culture

NOTE: The concentration factor takes into the account the exact numbers of cells used per reaction in relation to the number of cells/mL inferred from the $O.D._{600}$ measurement.

Cell culture conditions and transfection procedure

YT INDY T-cells were maintained in RPMI 1640 media containing 10% heat inactivated FCS, 0.1 mM MEM non-essential amino acids and 2 mM L-glutamine. Cells were diluted 1:4 every 2-3 days for maintenance.

Cells were transfected with DNA using the DEAE-dextran method. DEAE-dextran was prepared as a 10 mg/mL stock solution in TBS and filter sterilized. The GFP-CRT plasmid was generated by Nasrin Mesaeli. Briefly, to generate GFP-CRT targeted to the ER an oligonucleotide encoding for calreticulin's signal sequence was introduced upstream of the GFP coding sequence. Signal sequence oligonucleotides SS5 encoding the sense strand (5'-

TATAGCTAGCATGCTGCTCCCTGTGCCGCTGCTGCTCGGCCTGCTCGGCCTGGCCGCGCTAGCATAT-3') and SS3 encoding the antisense strand (5'-ATATGCTAGCGCGGCCAGGCCGAGCAGGCCGAGCAGCAGCGGCACAGGGAGCAGCATGCTAGCTATA-3') were annealed and cut with restriction enzyme *NheI* and ligated into the similarily cut and CIP treated p-S65T-C1 plasmid which encodes for the mutant form of GFP (Heim *et al.*, 1995). The generated plasmid encoding GFP with calreticulin's N-terminal signal, pS65T+LP, was confirmed by restriction digest and nucleotide sequencing. pGEX-CRT was digested with the restriction enzyme *EcoRI* to generate full length mature rabbit calreticulin. The gel purified calreticulin cDNA fragment was then ligated into the *EcoRI* digested and CIP treated pS65T+LP plasmid. Proper insertion of the calreticulin cDNA was confirmed by restriction endonuclease digestion.

Twenty four hours prior to transfection the cells were placed in 30 mL of fresh media per T75cm^2 flask. Prior to transfection the cell number was determined by counting using a hemocytometer. One million cells were aliquoted to a sterile 15 mL Falcon tube. The cells were pelleted by centrifugation at 1000 *x g* for 5 minutes followed by washing with sterile TBS. The cell pellet was resuspended in 1 mL of sterile TBS (1 x 10^6 cells/mL) and transferred to a fresh tube. Five μg of GFP-calreticulin plasmid DNA and 500 μg/mL of DEAE-dextran was added and mixed thoroughly. The transfection mixture was incubated at 37°C for 30 minutes and then centrifuged as before. The cell pellet was washed with complete media, centrifuged, resuspended in 10 mL of complete media and transferred to a T25 cm^2 flask. Cells were incubated at 37°C with 5% CO_2 in a humidified incubator for 48 hours followed by transfer to complete media containing 400 μg/mL G418. Cells were maintained in G418 for two weeks and the surviving cells examined for fluorescence.

Localization of GFP-calreticulin in T-cells

YT-INDY cells stable transfected with GFP-CRT expression vector were placed on poly-L-lysine coated coverslips (Harlow and Lane, 1988). The cells were allowed to attach for 15 minutes at room temperature followed by fixation with a 3.7% formaldehyde in PBS for 15 minutes. Coverslips were then mounted in Vinol 205S. Confocal microscopy was performed by Dr. M. Opas (University of Toronto).

Production of [^{35}S]-methionine labeled perforin

Full length, mature perforin was generated by restriction digest of pGAD-perf using *EcoRI* and *BamHI*. The transcription vector pGEM-3Z was also digested with *EcoRI* and *BamHI* endonucleases, CIP treated and purified. To generate the vector perforin-3Z (Fig. 2-10), the *EcoRI/BamHI* fragment encoding perforin was ligated into the pGEM-3Z vector resulting in the perforin cDNA being inserted down stream from the T7 RNA polymerase transcription initiation site (Fig. 2-10).

To produce ^{35}S labeled perforin the T7 TNT® Coupled Reticulocyte Lysate system was used (Promega). One μg of purified perforin-3Z plasmid DNA was mixed, on ice, with the buffer provided by the manufacturer and 50% TNT® reticulocyte lysate, 20 μM amino acid mixture minus methionine, 1.6 U/μL RNasin® ribonuclease inhibitor and 40 μCi of [^{35}S]-methionine. After thorough mixing 1 μL of the T7 RNA polymerase was added. The reaction was then incubated at 30°C for 60 minutes. Aliquots were removed at 30 and 60 minutes and analyzed by SDS-PAGE and autoradiography (Fig. 2-11). The resulting ^{35}S labeled perforin was used directly in the protein blotting experiments.

Protein blotting and ^{45}Ca^{2+} overlay

Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose using a semi-dry transfer apparatus. For protein blotting the membranes were incubated for 60

Figure 2-10: Structure of perforin-3Z

This is a schematic representation of the perforin-3Z cDNA clone constructed for the generation of radiolabeled perforin using the T7 TNT® Coupled Reticulocyte Lysate system. The cDNA of full length mouse perforin containing nucleotides 61-1665 was cloned into the *EcoRI* and *BamHI* restriction sites of the multiple cloning site (MCS) on the pGEM-3Z vector. The perforin cDNA was cloned downstream of a T7 promoter. The MCS is located within the lacZ reporter gene to allow for selection of transformants containing the perforin insert by blue/white screening of the bacterial colonies.

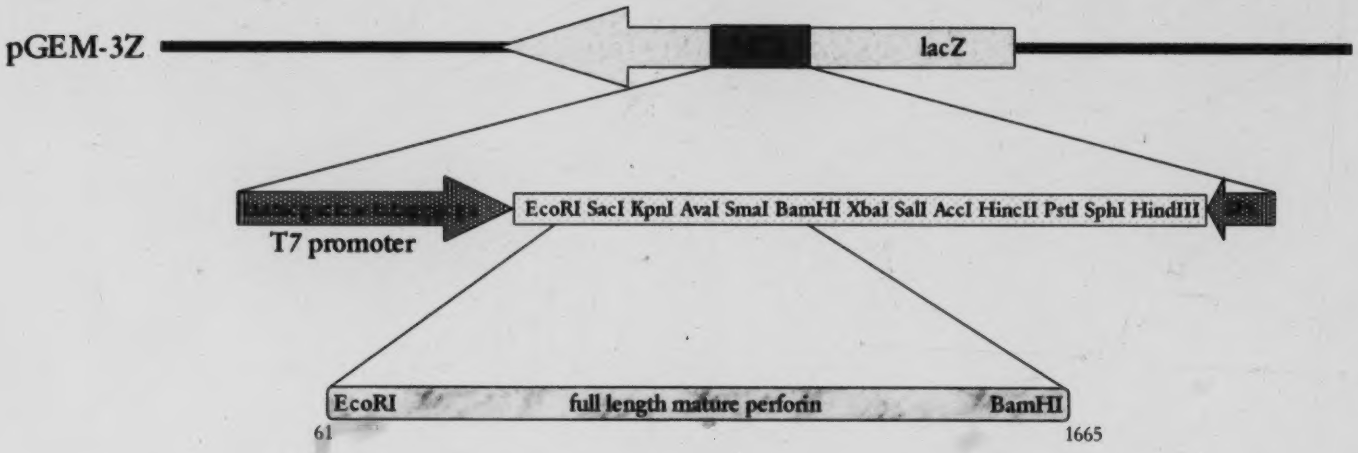
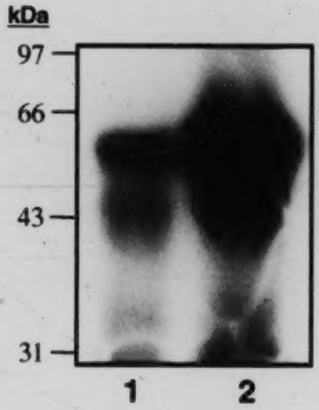

pGEM-3Z
lacZ
T7 promoter
EcoRI SacI KpnI AvaI SmaI BamHI XbaI SalI AccI HincII PstI SphI HindIII
EcoRI
full length mature perforin
BamHI
61
1665

Figure 2-11: [^{35}S]-methionine labeled expression of perforin

Radiolabeled perforin was expressed using the T7 TNT® Coupled Reticulocyte Lysate system in the presence of [^{35}S]-methionine. Aliquots of the reaction after 30 minutes (lane 1) and 60 minutes were run on 10% SDS-PAGE. The gel was incubated in Amplify (Amersham) for 60 minutes, dried and exposed to X-ray film for seven days. A 60 kDa radiolabeled band representing perforin was detected at both time points with a significantly higher expression after 60 minutes. Due to the generation of degradation products, the reaction was not allowed to proceed beyond 60 minutes. Molecular weight markers indicated are as follows: phophorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).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minutes with 5% BSA in blocking buffer containing 20 mM Tris-HCl, pH 7.0, and 150 mM KCl with or without 5 mM EGTA. Then, they were incubated with [^{35}S]-methionine labeled perforin in fresh blocking buffer (without BSA) for another 60 minutes. Unbound radioactively labeled perforin was washed away by three 60 minutes washes in blocking buffer. The blots were air dried overnight and exposed to X-ray film.

For $^{45}Ca^{2+}$ overlays the blot was subjected to three one hour washes in blocking buffer containing 60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole-HCl pH 6.8. The membrane was then incubated with 2.02 mCi/µl $^{45}Ca^{2+}$ in blocking buffer for 10 minutes. Unbound $^{45}Ca^{2+}$ was washed away with ddH_2O for 5 minutes. The membrane was allowed to air dry followed by exposure to X-ray film.

Immunoprecipitation

Purified CTL granules were solubilized in RIPA buffer (1% NP40, 0.5% DOC, 150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA and 1 mg/mL BSA in the presence or absence of 1.5 mM $CaCl_2$ (~600 µM free Ca^{2+}). Five µL of rabbit anti-calreticulin N1 antisera was added, mixed thoroughly and incubated at room temperature for 2 hours. Every step in the procedure was performed in the presence or absence of 1.5 mM $CaCl_2$. Fifty µL of 10% protein A/G beads, equilibrated in RIPA buffer containing 1 mg/mL BSA were added to the immunoprecipitation mixture and allowed to incubate for 30 minutes at room temperature. The beads were pelleted by centrifugation at 10,000 *x g* for 15 seconds. The beads were washed twice by resuspending in 500 µL of RIPA buffer (no BSA) and once with 500 µL 0.1% NP40, 25 mM Tris-HCl, pH 7.5 and 1 mM EDTA. The beads were resuspended in Laemmli sample buffer containing 2% SDS, heated for 2 minutes at 60°C and run on SDS-PAGE. The resulting gel was transferred to NC and analyzed by Western blotting with monoclonal anti-perforin antibodies.

CHAPTER THREE

EXPRESSION OF CALRETICULIN IN YEAST

INTRODUCTION

In the study of protein structure and function it is advantageous to have a reliable source of reasonable quantities of your protein of interest. Generally, proteins can be isolated in their native form from tissue sources but depending on the abundance and cellular location of the protein, the attainable yield and purity can pose difficulties. In response to the need for larger quantities of proteins for study, several expression systems have been developed. Many of these systems utilize bacteria for the protein production machinery due to its ease of handling and its rapid doubling time. Fusion proteins or tagged proteins can also be generated in these systems to simplify the purification procedures.

Although there are many advantages to these expression systems there are also drawbacks. Bacterial systems lack the protein processing, folding and modification machinery that eukaryotic cells have. Therefore, when producing a eukaryotic protein for structural or functional studies there is always a risk that proper folding may not have occurred or if working with systems that tag or fuse your protein of interest to another, the native structure may be altered. The result is that the data generated may not be physiologically relevant. As well, any essential post-translational modifications can not be studied.

The *Pichia pastoris* expression system overcomes many of these drawbacks while maintaining the ease of handling and the rapid production of the protein of interest. This system allows for the production of large quantities of the protein as well as its secretion into the external media for ease of isolation and purification (for reviews see Buckholz and

Gleeson, 1991; Cregg *et al.*, 1993; Sreekrishna *et al.*, 1988; Wegner, 1990). *Pichia pastoris* is very similar in nature to *Saccharomyces cerevisiae* in that it is easy to manipulate yet can have 10-100 fold higher levels of heterologous protein expression than its common counterpart. Also, being a eukaryotic cell, it provides the protein folding and post-translational modification machinery that bacterial expression systems lack. The *Pichia* system (Invitrogen) allows for either the intracellular or extracellular expression of the protein of interest. One major advantage to producing the protein as a secreted protein is that *Pichia* actually secretes very few endogenous proteins making the protein of interest a major component of the external media (Barr *et al.*, 1992).

The structural information known about calreticulin to date is based on predictions from the primary amino acid sequence but structural data has been lacking. In the interest of studying the structure and function of calreticulin I wanted to produce a sufficient quantity of calreticulin to attempt X-ray crystallography. Native calreticulin isolated from tissue sources or recombinant calreticulin fused to GST generated in *E. coli* can readily be purified by previously published methods (Baksh *et al.*, 1992; Baksh and Michalak, 1991) and have been used throughout this study. In both these methods degradation and yield of protein can be of concern. The GST-calreticulin fusion protein behaves in a similar manner to calreticulin with respect to its calcium binding (Baksh and Michalak, 1991) indicating that it is likely folded in a similar manner to the native protein. Unfortunately, attempts to crystallize the GST-calreticulin fusion protein has not been successful and therefore not suitable for structural studies. The goal of this study was to purify calreticulin in a relatively simple manner and in sufficient quantities to allow for X-ray crystallographic analysis of the protein. Importantly, yeast does not have any endogenous calreticulin or "calreticulin-like" protein and was therefore an appealing system in which to produce calreticulin. The *Pichia* *pastoris* expression system was therefore developed.

Pichia pastoris is a methylotrophic yeast that can utilize methanol as its sole carbon source. To deal with the hydrogen peroxide that is produced from metabolizing methanol the yeast produced alcohol oxidase. There are two genes coding for the alcohol oxidase, AOX1 and AOX2. AOX1 expression is tightly regulated by the presence of methanol. *Pichia* produces large amounts of alcohol oxidase in response to being grown on methanol and it is the AOX1 promoter that is mainly used to drive the over-expression of the protein of interest (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987). In this system the cDNA of the protein of interest is generated to be stably incorporated into the yeast genome by homologous recombination through either gene insertion or gene replacement at the AOX1 gene locus. In the event that gene replacement occurs the yeast will still grow on methanol due to the presence of the AOX2 gene but at a much slower rate.

Described here is the production of calreticulin in the Pichia expression system was established along with a purification procedure. The resulting protein was intended for structural studies but upon characterization some noteworthy observations were made. Very recently, in collaboration with Dr. James (University of Alberta) the yeast produced calreticulin has been crystallized and structural studies will hopefully be forth coming from his laboratory.

RESULTS

The pPIC9 expression vector contains the *Saccharomyces cerevisiae* α factor secretion signal to allow for secretion of the protein of interest into the media. Although several other secretion signals have been used with varying success the α factor signal has been the most successful (Cregg *et al.*, 1993; Scorer *et al.*, 1993). Therefore, pPIC9 was chosen for the cloning of calreticulin and expression in *Pichia* due to the potential ease of purification and the fact that calreticulin is normally localized to the ER (Fliegel *et al.*, 1989; Milner *et al.*, 1991; Michalak *et al.*, 1991; Opas *et al.*, 1991).

Expression of calreticulin in *Pichia*

Expression of calreticulin was induced by the inclusion of 0.5% methanol in the media. The protein produced in *Pichia* will be referred to throughout as yeast calreticulin. Both the external media and the cell pellet were analyzed by SDS-PAGE to determine the length of time required for optimum production of calreticulin. A large protein band with a mobility in SDS-PAGE corresponding to approximately 60 kDa was detected by Coomassie Blue staining at each time point (Fig. 3-1A). The highest level of expression was seen after 72 hours of growth at 30°C. The identity of the 60 kD band with calreticulin was verified by Western blot analysis using specific goat anti-calreticulin antibodies (Milner *et al.*, 1991) (Fig. 3-1B). Although there were detectable amounts of calreticulin being produced intracellularly only the secreted protein was isolated and purified due to its greater abundance and ease of handling. After determining the optimum conditions calreticulin production was scaled up and the resulting calreticulin purified from the media.

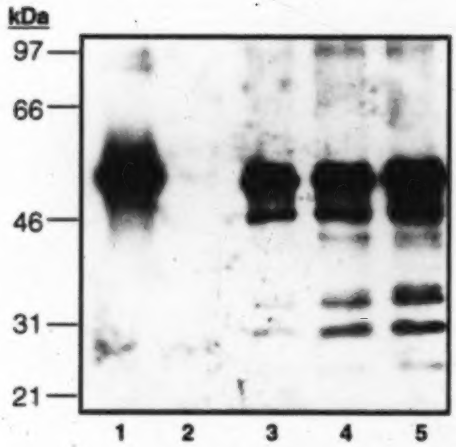
Purification of calreticulin

Essentially the first step in purifying calreticulin from yeast is done by having the protein secreted into the media. Calreticulin was isolated from the large scale culture by centrifuging to remove the yeast cells. The resulting media was then concentrated and the buffer exchanged by repeated washes with a low salt buffer using an Amicon concentrator with a 30,000 MW filter. Calreticulin was initially purified from the concentrated media by

Figure 3-1: Expression of Calreticulin in *Pichia*

The *Pichia* protein expression system (InVitrogen) was employed to generate full length CRT secreted into the growth media. The production of full length CRT in *Pichia pastoris* was induced by the addition of 0.5% methanol to the growth media every 24 hours. The secretion of CRT was analyzed by running aliquots of the growth media on 10% SDS-PAGE: lane 1, recombinant CRT; lane 2, blank; lane 3, 24 hours; lane 4, 48 hours; lane 5, 72 hours. CRT was detected by (A) Coomassie Blue staining or (B) Western blotting using goat anti-CRT antibodies (dilution 1:300). CRT is the major protein secreted by the yeast cells and the highest level of CRT expression achieved without significant degradation was after 72 hours. Molecular weight markers indicated are as follows: phophorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500).

A.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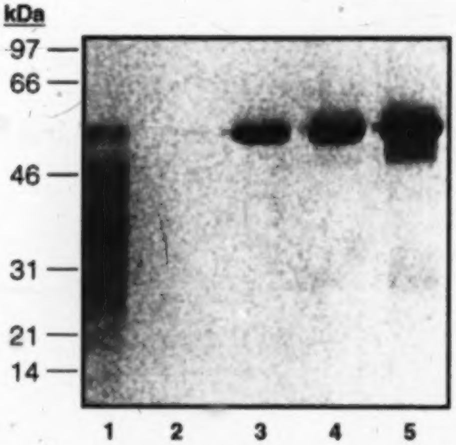
B.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DEAE-sephadex chromatography. This purification step allowed for the removal of media components from the calreticulin protein and resulted in a partially purified product. The DEAE calreticulin fractions were further purified using another ion exchange column, the Resource Q (Pharmacia) column on the FPLC due to its ease of handling and high capacity (Fig. 3-2A). Fractions were collected and analyzed by SDS-PAGE (Fig. 3-2B). Coomassie Blue staining of the gel revealed that fractions eluted at 57% B (450 mM NaCl) were approximately 95% pure. These fractions were pooled and concentrated. Yields obtained were between 4-6 mg of purified protein per 1 L of culture.

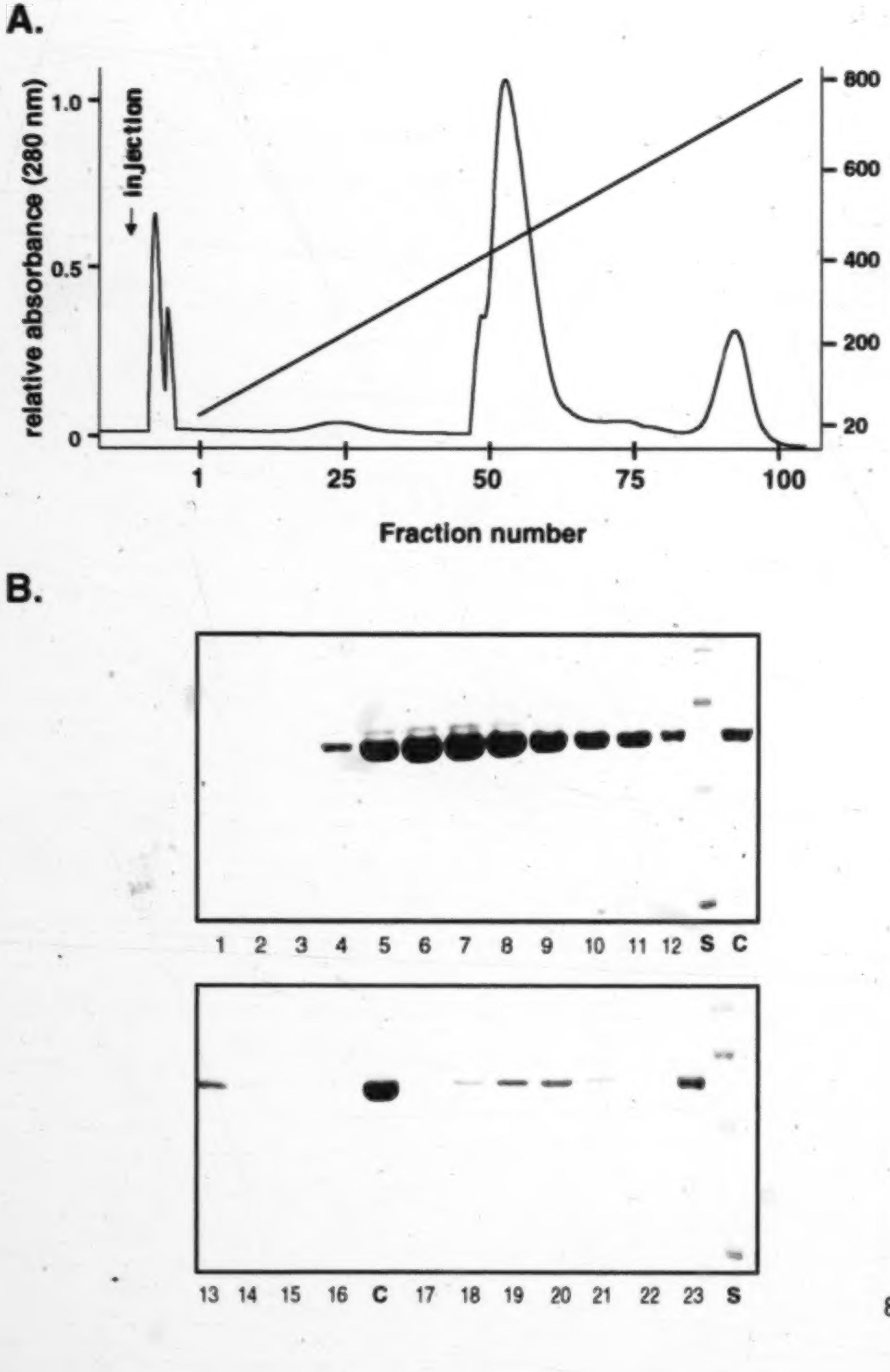
Characterization of calreticulin produced in yeast

Ample quantities of calreticulin were produced in this system and were secreted into the media despite the fact that the cDNA of calreticulin used in these experiments encodes for the C-terminal KDEL amino acid sequence known to be responsible for retention of proteins in the ER (Pelham, 1989). To determine if the KDEL amino acid sequence was present on the yeast calreticulin, the protein was analyzed by Western blot using a calreticulin specific antibody, CRT283 (discussed in Chapter 5), that recognizes the peptide sequence QAKDEL. Figure 3-3 indicates that yeast calreticulin produced does in fact have a KDEL ER retrieval signal like its counterpart isolated from tissue. This suggests that the calreticulin produced is full-length and does not lose its ER retrieval signal during the secretion process.

The rabbit calreticulin cDNA used for the generation of the pPIC-calreticulin expression vector encodes a putative N-glycosylation site at amino acid 327. Although rabbit calreticulin is not glycosylated, the yeast calreticulin was examined for possible glycosylation. Concanavalin A (ConA) can be used to detect glycosylated proteins due to its ability to bind to carbohydrate chains. Digestion of glycosylated proteins with

Figure 3-2: Resource Q chromatogram and fractions

CRT produced in the Pichia expression system was initially purified using DEAE sepharose chromatography. CRT fractions collected from the DEAE sepharose column were further purified using a 6 mL Resource Q column (Pharmacia) run on the FPLC. **A.** The chromatogram of CRT eluted from the Resource Q column using a high salt gradient of 780 mM NaCl in 20 mM NaH_2PO_4, Na_2HPO_4 pH 7. **B.** One mL fractions were collected and run on 10% SDS-PAGE and visualized by Coomassie Blue staining. Lanes 1 – 13 represent aliquots taken from every second fraction collected while the gradient was held at 57% B (450mM NaCl). Lanes 14 – 23 represent aliquots taken from every second fraction collected from 57% - 72% B. Lane (C), recombinant CRT, as a control; lane (S), low standard molecular weight markers visualized are as follows: phophorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700) and bovine carbonic anhydrase (31,000). The majority of purified CRT eluted at 57% B.


A.
relative absorbance (280 nm)
1.0
0.5
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← injection
[NaCl] mM
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600
400
200
20
1
25
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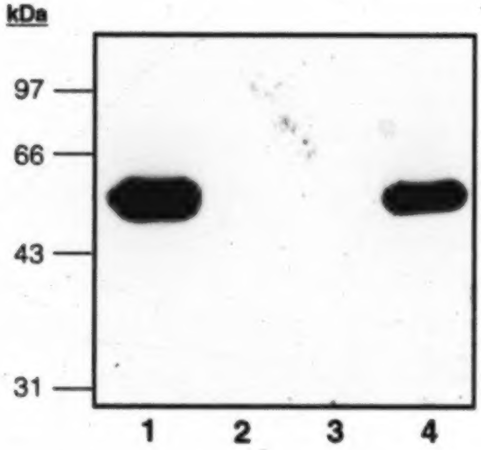
75
100
Fraction number
B.
1 2 3 4 5 6 7 8 9 10 11 12 S C
13 14 15 16 C 17 18 19 20 21 22 23 S

Figure 3-3: Detection of KDEL retention signal

Due to the secretion of CRT produced in *Pichia* the presence of CRT's KDEL retention signal was evaluated. A western blot was performed using CRT283 antibody that specifically recognizes the C-terminal KDEL retention signal of CRT. Lane 1, dog pancreatic CRT; lane 2, bovine serum albumin (BSA); lane 3, canine cardiac calsequestrin; and lane 4, purified yeast CRT from *Pichia*. Samples were run on 10% SDS-PAGE, transferred to nitrocellulose and western blotted with CRT283 antibody (1:100 dilution). Antibody binding was visualized chemiluminescence. Molecular weight markers indicated are as follows: phophorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).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endoglycosidases also allows for the verification of the presence of carbohydrate chains on the protein. Glycosylation of yeast calreticulin was detected by running the protein and endoglycosidase digests on SDS-PAGE, transferring to nitrocellulose and then performing a protein overlay with ConA (Fig. 3-4). Cardiac calsequestrin (CCS) and dog pancreatic calreticulin were also digested and run in parallel as positive and negative controls, respectively (Fig. 3-4). CCS is highly glycosylated protein with high-mannose type oligosacchardies and clearly shows a marked reduction of sugar detected after endo H digestion as compared with untreated CCS (Fig. 3-4, lanes 4 and 5) indicating the effectiveness of the enzyme. The dog pancreatic protein reveals a faint band in each lane, with or without digestion by the endoglycosidases indicating non-specific binding of the Con A (Fig. 3-4, lanes 8-11). The yeast calreticulin clearly shows ConA binding indicating that it is glycosylated (Fig. 3-4, lane 12). After endo H digestion of the yeast calreticulin ConA no longer binds the protein indicating that the sugar has been completely cleaved. This further confirms the fact that the yeast calreticulin is glycosylated. The majority of glycosylation events in *Pichia* are N-linked and of the high-mannose type averaging 8-14 mannose residues per side chain (Tschopp *et al.*, 1987b). Therefore, the putative glycosylation site in the rabbit amino acid sequence is a functional glycosylation signal.

The calcium binding property of calreticulin was one of the first features discovered about the protein (Ostwald and MacLennan, 1974; Michalak *et al.*, 1980) and is a standard characteristic of the protein. The yeast calreticulin was then examined for its ability to bind calcium using the $^{45}Ca^{2+}$ overlay technique. CCS and recombinant calreticulin, purified from bacteria (Baksh *et al.*, 1992), as expected showed calcium binding (Fig. 3-5, lanes 2 and 4 respectively). BSA was also included as a negative control and showed no calcium binding

Figure 3-4: Glycosylation of Calreticulin

The glycosylation state of CRT produced in *Pichia* was evaluated by endoglycosidase digestion and protein overlay with Concanavalin A (ConA). Yeast CRT was digested with endoglycosidase H (endoH) or endoglycosidase F (endoF). Cardiac calsequestrin (CS) and dog pancreatic CRT (DP CRT) were also digested as controls. Undigested and digested samples were run on 10% SDS-PAGE, transferred to nitrocellulose and stained with horseradish peroxidase conjugated ConA. ConA binding was visualized by chemiluminescence. Lane 1, prestained molecular weight standards; lane 2, undigested CS; lane 3, endoH digested CS; lane 4, endoF digested CS; lane 5, blank; lane 6 and 7, undigested DP CRT; lane 8, endoH digested DP CRT; lane 9, endoF digested DP CRT; lane 10, undigested yeast CRT; lane 11, endoH digested yeast CRT; lane 12, endoF digested yeast CRT. Molecular weight markers indicated are as follows: phosphorylase b (101,000), bovine serum albumin (83,000), ovalbumin (50,600), carbonic anhydrase (35,500).

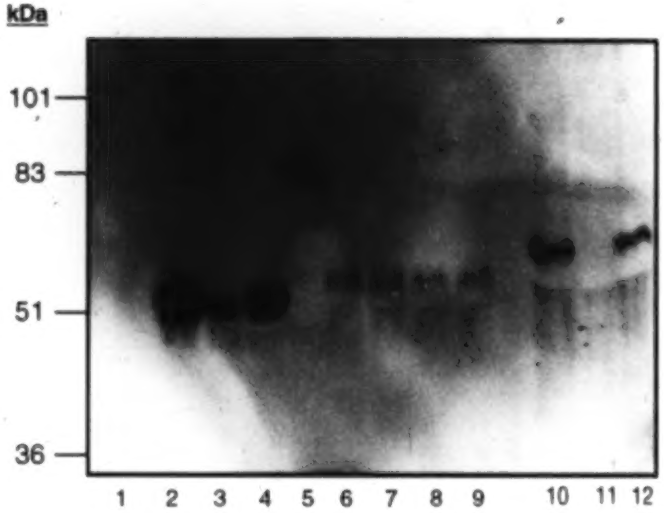
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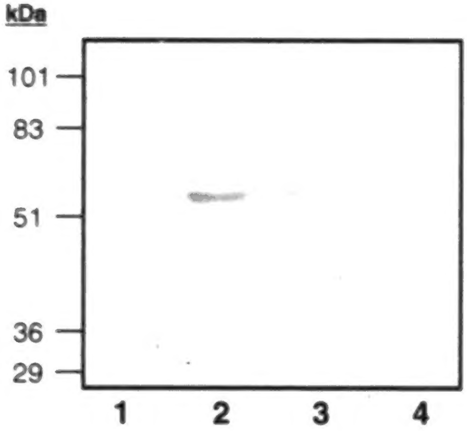
Figure 3-5: $^{45}Ca^{2+}$ overlay

The ability of yeast produced CRT to bind calcium was determined by the $^{45}Ca^{2+}$ overlay technique. Samples (3 μg/lane) were run on 10% SDS-PAGE and transferred to nitrocellulose. The blot was blocked prior to incubation with 2.02 mCi/μL of $^{45}Ca^{2+}$. Excess radiolabeled Ca^{2+} was removed by washing. Bound Ca^{2+} was detected by autoradiography. Lane 1, BSA; lane 2, CS; lane 3, recombinant CRT; lane 4, yeast CRT. Molecular weight markers indicated are as follows: phosphorylase b (101,000), bovine serum albumin (83,000), ovalbumin (50,600), carbonic anhydrase (35,500), soybean trypsin inhibitor (29,100).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(Fig. 3-5, lane 1). The yeast calreticulin also bound $^{45}Ca^{2+}$ (Fig. 3-5, lane 3). Yeast calreticulin binds calcium indicating that its structure is similar to that of calreticulins isolated from other sources. As well, this indicates that the glycosylation of yeast calreticulin has not altered the calcium binding properties of the protein.

Generation and characterization of anti-calreticulin antibodies

Western blot analysis and immunocytochemistry are just two valuable techniques for studying proteins that require specific antibodies to the protein of interest. The production of calreticulin in *Pichia* provided ample quantities of the purified protein to attempt generating specific antibodies to the yeast calreticulin. The protein was injected into rabbits at regular intervals. The resulting serum was tested via Western blot analysis for its ability to bind to calreticulin. The obtained antibodies recognized both native calreticulin present in pancreatic microsomes as well as the yeast produced calreticulin (Fig. 3-6, lanes 1 and 3 respectively). The antibodies were also analyzed for their ability to recognize GST-fusion proteins with either the N-, P-, or C-domain. The anti-yeast calreticulin antibodies strongly recognize the GST-P domain of calreticulin (Fig. 3-6, lane 5) but did not recognize the GST-N domain and very weakly the GST-C domain. The antibodies did not recognize GST alone (Fig. 3-6, lane 7). Therefore, the antibodies generated against the yeast calreticulin protein recognize calreticulin from other sources and specifically recognize the P-domain of the protein.

The antibodies generated were also examined using indirect immunocytochemistry. RPE-Y cells stained with anti-yeast calreticulin antibodies revealed a punctate like pattern of staining (Fig. 3-7A). The network of calreticulin detected throughout the cell is typical of proteins localized to the ER and has been seen before in other cell types for calreticulin (Michalak *et al.*, 1991). As a control, the RPE-Y cells were also stained with goat anti-

Figure 3-6: Specificity of newly generated anti-CRT antibodies

Antibodies were generated to yeast CRT by inoculating rabbits at regular time intervals with the purified yeast CRT. The resulting serum contained CRT specific antibodies. The antibodies were characterized by examining their ability to recognize the different domains of CRT fused to GST. Samples were run on 12.5% SDS-PAGE, transferred to nitrocellulose and western blotted with the generated antibodies (dilution 1:5000). Lane 1, pancreatic microsomes; lane 2, ER microsome preparation; lane 3, yeast CRT; lane 4, GST-N; lane 5, GST-P; lane 6, GST-C; lane 7, GST alone. Molecular weight markers indicated are as follows: phosphorylase b (101,000), bovine serum albumin (83,000), ovalbumin (50,600), carbonic anhydrase (35,500), soybean trypsin inhibitor (29,100), lysozyme (20,900).

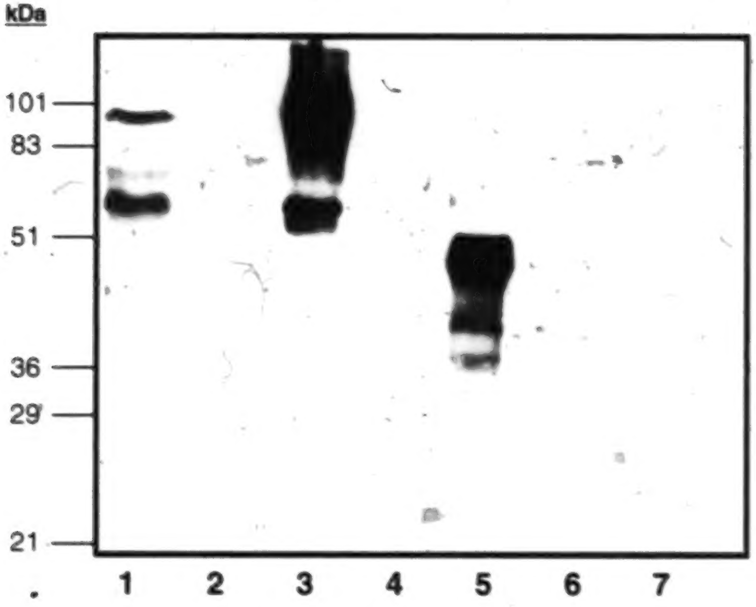
kDa
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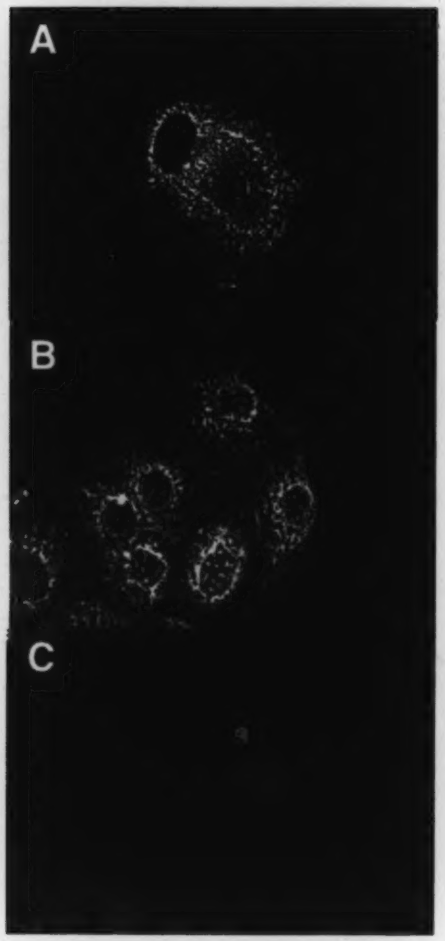
Figure 3-7: Immunostaining of RPE-Y cells

The newly generated antibodies to purified yeast CRT were examined for their usefulness in immunocytochemistry. RPE-Y cells were grown on coverslips, fixed, permeablized and stained with CRT specific antibodies or preimmune serum. Panel A was stained with the newly generated antibodies to yeast CRT; Panel B was stained with goat anti-CRT antibodies and Panel C was stained with preimmune serum. A punctate pattern indicative of ER localization was detected with the newly generated antibodies similar to that detected with the goat anti-CRT antibodies. No staining was detected with the preimmune serum.


A
B
C

calreticulin antibodies (Milner *et al.*, 1991) and preimmune serum (Fig. 3-7B and C, respectively). The goat antibody revealed a punctate like pattern indicating the ER localization of calreticulin. No staining was detected with the preimmune serum. The rabbit

DISCUSSION

This study successfully established the *Pichia* expression system to produce large quantities of calreticulin and a rapid purification procedure. By design, the *Pichia* system allowed for the production of calreticulin as a secreted protein. Due to the fact that *Pichia* secretes very few proteins into the external media (Barr *et al.*, 1992), this served as an initial purification step greatly simplifying the overall purification process. Further purification of yeast calreticulin was achieved by the use of two ion-exchange columns in succession that exploit the highly acidic nature of calreticulin (Michalak *et al.*, 1992; Smith and Koch, 1989). The purification procedure developed here is both simple and relatively quick making this an appealing way to produce calreticulin for further study. The *Pichia* system, therefore, allows for the reliable and reproducible production of ample quantities of calreticulin.

Structural information available about calreticulin has been predicted from its amino acid sequence (Michalak *et al.*, 1992; Smith and Koch, 1989) but structural data pertaining to calreticulin has been lacking. The availability of large quantities of yeast calreticulin now provides an avenue for studying structural components of calreticulin. The large quantities of calreticulin produced in yeast now available are being used for X-ray crystallograpic analysis. Previous attempts to crystallize calreticulin from other sources were unsuccessful but yeast produced calreticulin very recently has been crystallized in Dr. James' laboratory (University of Alberta). Attempts to determine if the yeast calreticulin crystals will defract are underway. In the future, with the *Pichia* system well established, the production of the various domains of calreticulin may be possible. This would then allow structural studies of the individual sections of calreticulin in comparison with the full length protein. The possibility of examining structural changes through NMR and CD spectroscopy in response to ion binding will also be important.

Upon purifying yeast calreticulin some interesting and unexpected observations were made. An important feature of calreticulin is its C-terminal KDEL sequence (Sonnichsen *et al.*, 1994). The -KDEL amino acid sequence is known to be responsible for localizing proteins to the ER (Pelham, 1989). Not surprisingly, calreticulin has been found to be localized to the ER in a variety of cell types (Fliegel, *et al.*, 1989; Milner *et al.*, 1991; Michalak *et al.*, 1991; Opas *et al.*, 1991). Even though yeast produced calreticulin was successfully secreted the protein was clearly shown to have an intact KDEL amino acid sequence. This would indicate that the yeast calreticulin produced is full-length and does not lose the KDEL ER retrieval signal during the secretion process. It is unknown whether the yeast calreticulin KDEL retention signal is functional. It is possible that the KDEL retention signal is somehow masked and not recognized by the retention/retrieval machinery but some yeast calreticulin is located in the cell and may be due to retention via the KDEL sequence. Another possibility is that the yeast calreticulin is secreted as a stress response due to overloading the ER with the overexpressed protein.

There are indications that calreticulin is capable of escaping the ER. One example is its localization to the cytolytic granules of cytotoxic T lymphocytes (Dupuis *et al.*, 1993) (discussed further in Chapter 5). Native calreticulin has been found at extracellular locations (Eggleton *et al.*, 1994; Gray *et al.*, 1995; Sueyoshi *et al.*, 1991, White *et al.*, 1995). Full length calreticulin with its KDEL ER retrieval signal has been found to be released from human neutrophils (Eggleton *et al.*, 1994) as well as being present in low levels in human plasma

(Sueyoshi *et al.*, 1991). The role that calreticulin may be playing in these locations is not fully understood. There is also an example of ER resident, KDEL containing protein, GRP94 being detected on the cell surface (Altmeyer *et al.*, 1996; reviewed in Nicchitta, 1998). How this protein escapes the ER is also not known. Cell surface calreticulin has been implicated in affecting cell spreading through its lectin like activity (Gray *et al.*, 1995). Also, there is evidence that calreticulin may be involved in the inhibition of thrombotic and inflammatory responses (Kuwabara *et al.*, 1995). Another very important finding was the fact that arterial infusion of calreticulin before angioplasty reduced restenosis (Dai *et al.*, 1997). These findings indicate that calreticulin may be useful for a clinical application and a rapid, simple procedure for the isolation of large quantities of calreticulin would be an asset.

The glycosylation of calreticulin has been somewhat puzzling. Glycosylation of calreticulin has only been detected in the protein isolated form bovine brain, rat liver, Chinese hamster ovary cells, *Leishmania* and spinach (Waisman *et al.*, 1985; Van *et al.*, 1989; Jethmalani *et al.*, 1994; Matsuoka *et al.*, 1994; Joshi *et al.*, 1996; Navazio *et al.*, 1996). These glycosylation events were also somewhat different. The glycosylation of calreticulin from bovine brain occurs at an alternative site from the putative asparagine residue #327 (Matsuoka *et al.*, 1994), while the rat liver calreticulin contained an unusual complex sugar (Van *et al.*, 1989). The glycosylation of calreticulin from Chinese hamster ovary cells occurs in a "prompt glycosylation" response to heat stress (Jethmalani *et al.*, 1994). Although rabbit calreticulin encodes for a putative N-linked glycosylation site the native protein is not glycosylated (Michalak *et al.*, 1992). A very important feature discovered about the yeast calreticulin, produced from the rabbit cDNA, is that it is glycosylated as determined by ConA binding and endoglycosidase digestion. The fact that the yeast calreticulin produced is glycosylated indicates that the putative site is functional and recognized by the yeast glycosylation machinery. Why is it that the yeast calreticulin is glycosylated while the native rabbit protein is not? The reason for the discrepancy is not clear. Differences in the glycosylation machinery between yeast and other higher eukaryotes has not been unveiled. Clearly this is another example of species specific glycosylation of calreticulin. The functional significance of having calreticulin glycosylated and how it might affect calreticulin-protein interactions is not understood. The yeast calreticulin now provides a tool for studying the importance of the glycosylation of calreticulin.

An important feature of calreticulin is its ability to bind calcium. The yeast protein was examined for its ability to bind calcium to ensure that it behaves in a similar manner to calreticulin isolated from other sources. The yeast calreticulin is able to bind calcium as detected by the $^{45}Ca^{2+}$ overlay technique. This indicates that the yeast calreticulin is likely properly folded. It also implies that the glycosylation of the protein does not interfere with the protein's ability to bind calcium.

The yeast calreticulin produced proved to be antigenic upon injection into rabbits and resulted in antibodies specific for the P-domain of calreticulin. The P-domain of calreticulin has been shown to have functional significance. The P-domain has been shown to be involved in calreticulin interacting with PDI (Baksh *et al.*, 1995) as well as being involved in calreticulin's binding to perforin (Andrin *et al.*, 1998) (discussed in Chapter 5). The P-domain contains highly conserved, repetitive amino acid sequences (Chapter 1) that are homologous to amino acid repeats found in calnexin, calmegin and CALNUC (Milner *et al.*, 1991; Bergeron *et al.*, 1994; Lin *et al.*, 1998). These repeats are involved in the lectin binding site of each of these proteins (Vassilakos *et al.*, 1998) suggesting that this region is then essential for their chaperone activities. The P-domain is also a site of high affinity calcium binding (Baksh and Michalak, 1991). With the functional significance of this region of the protein it will be especially useful to have specific antibodies to this domain and there are no other P-domain specific antibodies available that we are aware of. Previously made goat anti-calreticulin antibodies resulted in antibodies mainly specific for the C-domain of the protein (Milner *et al.*, 1991). Possibly the glycosylation of the yeast calreticulin, presumably in the C-domain, results in subtle conformational changes that result in the P-domain being exposed and more antigenic.

SUMMARY

In this study the *Pichia* expression system was established for producing large quantites of calreticulin for structural studies. A rapid, simple purification scheme was devised and resulted in high yields of reasonably pure protein. Characterization of the protein revealed that it contains an intact KDEL ER retrieval signal and binds calcium as expected. Surprisingly, the yeast calreticulin was found to be glycosylated. This is of major importance since the native rabbit calreticulin is not glycosylated. As well, P-domain specific antibodies were generated indicating that the P-domain is the most antigenic in the yeast calreticulin and that this region must be more exposed than in other calreticulins. These antibodies are of significance due to the functional importance of this domain of calreticulin and the fact that there are no other P-domain specific antibodies available that we know of. Overall, establishing the *Pichia* expression system has provided a reliable source of calreticulin for further structural and functional studies. Specifically, how the role of glycosylation of calreticulin might affect calreticulin-protein interactions and functional aspects of the protein may be addressed.

CHAPTER FOUR

INTERACTION OF CALRETICULIN WITH THE GLUCOCORTICOID RECEPTOR

A version of this chapter has been published:
Baksh, S., Burns, K., Andrin, C., Michalak, M. 1995. **Interaction of calreticulin with protein disulfide isomerase.** J. Biol. Chem. 270: 31338-31344
Michalak, M., Burns, K., Andrin, C., Mesaeli, N., Jass, G. H., Busaan, J., Opas, M. 1996. **Endoplasmic reticulum form of calreticulin modulates glucocorticoid-sensitive gene expression.** J. Biol. Chem. 271: 29436-29445

INTRODUCTION

When this study started it was hypothesized that calreticulin must gain access to the nucleus and be localized there at least transiently based on several findings. Calreticulin binds to a conserved amino acid sequence, K-L-G-F-F-K-R, found in the cytoplasmic domain of all integrin α-subunits (Roajiani *et al.*, 1991). This peptide sequence is also found in the DNA binding domain of receptors belonging to the nuclear hormone receptor superfamily (Evans, 1988; Beato, 1989; Laudet *et al.*, 1992; Luisi *et al.*, 1991). This, along with the fact that calreticulin had been localized to the nucleus and the nuclear envelope of some cells (Michalak *et al.*, 1992), indicates that calreticulin may be interacting with nuclear hormone receptors and affecting their function. Calreticulin's role in the modulation of steroid sensitive gene expression has now been well established (Burns *et al.*, 1994; Dedhar *et al.*, 1994; Winrow *et al.*, 1995; Desai *et al.*, 1996; Shago *et al.*, 1997). Although a calreticulin "like" antigen has been localized to the nucleus (Opas *et al.*, 1991; Roderick *et al.*, 1997) and associates with α intergrins (Rojiani *et al.*, 1991; Coppolino *et al.*, 1995), implying a cytoplasmic localization, there is still much confusion around how calreticulin might escape the retention/retrieval mechanisms of the ER. This led us to investigate the protein-protein interactions between calreticulin and the GR as well as examining the potential nuclear localization of calreticulin. Calreticulin's ability to affect glucocorticoid sensitive gene expression from either an ER localization or a cytoplasmic localization was also examined.

The GR belongs to a subfamily of the nuclear hormone receptor superfamily known as the steroid receptors (for complete review see Tsia and O'Malley eds., 1994). The nuclear hormone receptors are involved in regulating development, morphogenesis, reproduction, behavior and homeostasis (Tsia and O'Malley eds., 1994). These receptors are ligand dependent transcription factors that can activate transcription upon hormone binding. The steroid receptors of the nuclear hormone receptor superfamily include the corticosteroid receptors which can be divided into the glucocorticoid and mineralcorticoid receptors; progesterone, androgen and estrogen receptors (Tsia and O'Malley eds., 1994). The GR has been implicated in the transcriptional control of many different genes affecting several metabolic pathways and systems (Tsia and O'Malley eds., 1994). It is specifically involved in the regulation of carbohydrate, protein and lipid metabolism as well as affecting the cardiovascular, renal and nervous systems (Tsia and O'Malley eds., 1994).

The GR is believed to be located in an inactive state in the cytoplasm and upon hormone binding undergoes dimerization and nuclear translocation (Antakly and Eisen, 1984; Govindan, 1980; Papamichail *et al.*, 1980; Wilkstrom *et al.*, 1987). The cytoplasmic GR is found complexed with heat shock proteins, hsp90, hsp70 and hsp56 (Rexin *et al.*, 1992) which are thought to help maintain the receptor in its inactive state. Upon translocation to the nucleus the GR is able to bind to its specific GRE, as a homodimer, in a hormone dependent manner (Becker *et al.*, 1986) and activates transcription.

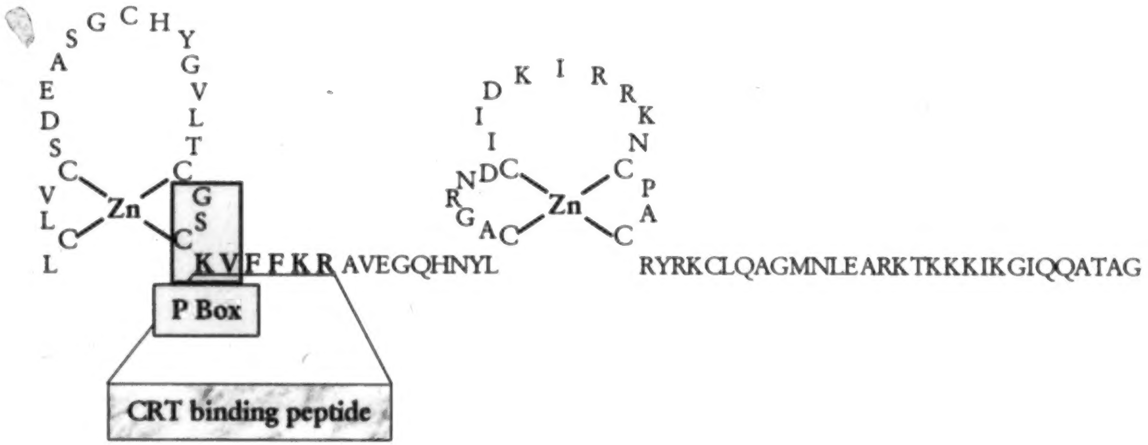
The GR can be divided into three structural domains: the NH_2-domain, the DNA binding domain and the ligand binding domain (Giguere *et al.*, 1986; Beato, 1989). It is important to note that these domains are self contained functional units that can be fused to other proteins and still retain their function (Evans, 1988). The DNA binding domain located in the central part of the molecule is the most conserved whereas the NH_2-domain is more variable. The ligand binding domain is located in the carboxy terminal portion of the molecule and confers specificity of hormone binding to the receptor.

The DNA binding domain contains two zinc finger modules that are highly conserved (Freedman *et al.*, 1988; Evans and Hollenberg, 1988). The K-X-F-F-K/R-R consensus amino acid sequence is located between the two zinc fingers at the base of the first zinc finger. The initial K residue is part of the region coined the P Box (Fig. 4-1) that confers specificity of the receptor to its HRE (Umesono and Evans, 1989; Danielson *et al*, 1989; Mader *et al.*, 1989). This finding was supported by the X-ray crystallographic data of the GR DNA binding domain bound to its GRE which showed that the conserved peptide sequence (K-V-F-F-K-R for the GR) was involved in DNA binding (Luisi *et al.*, 1991). It forms an alpha helix that is the direct contact with the DNA (Luisi *et al.*, 1991).

Calreticulin interacts with the GR DNA binding domain *in vitro* (Burns *et al.*, 1994). This binding is directly through the K-V-F-F-K-R amino acid sequence located in the GR DNA binding domain as determined by peptide competition experiments (Burns *et al.*, 1994). Therefore, if calreticulin interacts directly with the GR *in vivo* it would interfere with the GR's ability to bind to its cognate GRE. Calreticulin also interacts with other nuclear hormone receptors such as the androgen receptor DNA binding domain (Dedhar *et al.*, 1994), the vitamin D receptor binding domain, full-length vitamin D receptor/retinoid X receptor heterodimers (Wheeler *et al.*, 1995), retinoic acid receptor (Desai *et al.*, 1996) and retinoic acid receptor/retinoid X receptor heterodimers (Shago *et al.*, 1997). In each case the calreticulin-receptor interaction was competed with peptide indicating the specificity of the interaction with the K-V-F-F-K-R amino acid sequence. The functional significance of these interactions has also been shown *in vivo*. Overexpression of calreticulin inhibits the activation of reporter gene expression under the control of different hormone receptors in various cell systems (Burns *et al.*, 1994; Dedhar *et al.*, 1994; Wheeler *et al.*, 1995; Desai *et al.*, 1996). In each of these studies calreticulin was expressed as full-length protein and therefore

Figure 4-1: DNA binding domain of the glucocorticoid receptor

This is a schematic diagram of the amino acid sequence of the DNA binding domain of the GR. The zinc ions shown are tetrahedrally coordinated to four cysteine residues forming a classical zinc finger motif. The P-box, involved in the DNA binding specificity, is highlighted in grey. The highly conserved KVFFKR amino acid motif involved in DNA binding is shown in bold and underlined. This peptide motif is also known to bind to CRT *in vitro* as indicated.


S G C H
A Y
E G
D V
S L
C T
V C
Zn
G
S
L C
C
L
K V F F K R AVEGQHNYL
P Box
CRT binding peptide
D K I R
I R
I K
N D C N
R C
Zn P
G A C A
C
RYRKCLQAGMNLEARKTKKKIKGIQQATAG

presumably was located in the ER. The question then remains as to how calreticulin manages to escape the ER retention/retrieval mechanisms to gain access to the various receptors, bind to them and inhibit there function *in vivo*.

RESULTS

Immunostaining of intact nuclei with different calreticulin antibodies

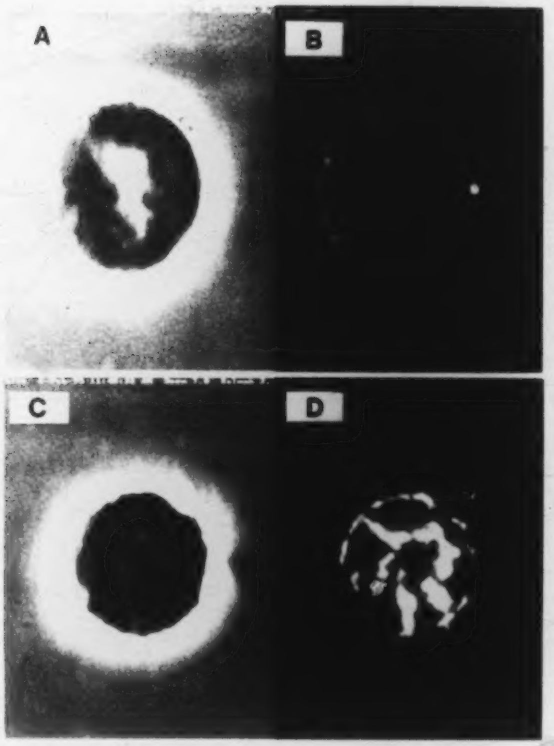
To determine if calreticulin is in fact a nuclear resident protein, isolated nuclei from rat liver were examined by immunostaining with specific anti-calreticulin antibodies. Pure intact nuclei were allowed [illegible] attach to poly-L-lysine coated coverslips and permeablized. Two different antibodies were used, the goat anti-calreticulin antibodies or rabbit anti-calreticulin antibodies. The immunostained nuclei were then examined by confocal microscopy (Fig. 4-2). Immunostaining with the goat anti-calreticulin antibodies did not reveal any nuclear calreticulin in the isolated nuclei(Fig. 4-2B). However, the rabbit anti-calreticulin antibodies did reveal patches of nuclear staining (Fig. 4-2D). The reason for the discrepancy between the results obtained with the different antibodies is not clear at present. The nuclear staining observed with the rabbit anti-calreticulin antibodies may be artifactual. It may also be that the different isolated nuclei are contaminated with different amounts of nuclear envelope membrane.

Examination of fractionated nuclei for the presence of calreticulin

To further examine the possibility of a nuclear calreticulin we fractionated intact nuclei into outer nuclear membrane and inner nuclear membrane/nuclear content fractions followed by Western blot analysis for the presence of calreticulin. Fractionation of purified nuclei was achieved by extracting with 5% Triton X-100. This results in a detergent soluble

Figure 4-2: Immunostaining of intact nuclei

Pure, intact nuclei were attached to poly-L-lysine coated coverslips, fixed and permeablized before immunostaining with CRT specific antibodies to determine if any CRT was localized to the nucleus. Panel B was stained with goat anti-CRT sera while Panel D was stained with rabbit anti-CRT sera. Panels A and C are phase contrast pictures of the isolated nuclei, respectively. CRT staining was only detected with the rabbit anti-CRT antibodies indicating a discrepancy between the results obtained from the two CRT specific antibodies.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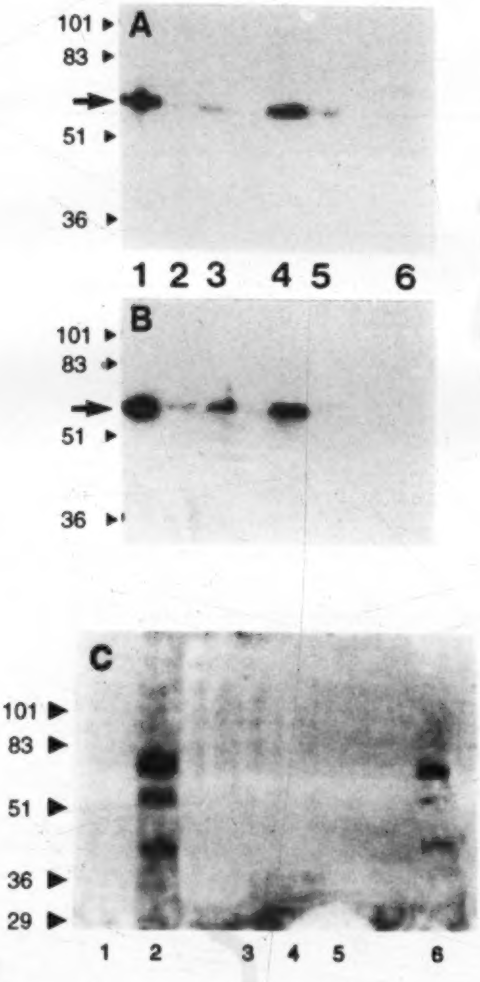
fraction containing the outer nuclear membrane and an insoluble fraction containing the contents of the nuclei surrounded by the inner nuclear membrane. Resident nuclear proteins are contained within the insoluble fraction and proteins associated with the soluble fraction are generally of ER origin. The fractions were analyzed by Western blot with either the goat anti-calreticulin antibodies (Fig. 4-3A), rabbit anti-calreticulin antibodies (Fig. 4-3B) or as a control, antibodies to the lamin-B receptor (Fig. 4-3C), an integral inner nuclear membrane marker protein. Fig. 4-3 A and B both show that calreticulin is present in intact nuclei (lane 3) but upon fractionation calreticulin is only associated with the detergent soluble, outer nuclear membrane (lane 4) but not with the insoluble, inner membrane containing nuclei (lane 6). Fig. 4-3 C shows that the lamin-B receptor is detected in purified, intact nuclei (lane 2) and after fractionation is only associated with the insoluble inner nuclear membrane and nuclear contents (lane 6). This indicates that the soluble nuclear fraction in this experiment is not contaminated with inner nuclear membrane. These results show that calreticulin is not a resident nuclear protein and its detection in the nucleus is likely due to its presence in the outer nuclear membrane fraction which is of ER origin.

DNA mobility shift analysis of calreticulin and glucocorticoid receptor interactions

Calreticulin interacts with the DNA binding domain of the GR *in vitro* (Burns et al, 1994). The DNA mobility shift assay was employed to verify this result and to examine whether calreticulin is capable of interacting with the GR when it is already bound to its GRE. The DNA mobility shift allows for the detection of proteins that are capable of binding to oligonucleotide. In this case, the DNA binding domain of the GR was incubated with an excess of its cognate radiolabeled GRE followed by non-denaturing gel electrophoresis. The GR-GRE complex is detected as a radiolabeled band that has shifted to a slower mobility in comparison to the unbound radiolabeled oligonucleotide. Putative

Figure 4-3: Nuclear fractionation

Pure, isolated nuclei were fractionated with Triton X-100. Purified nulcei were washed with 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl pH 7.5. The nuclei were solubilized with 5% Triton X-100 followed by centrifugation at 800 x *g*. The Triton X-100 supernatant contains solubilized outer nuclear membrane whereas the pellet contains the intact inner nuclear membrane and the nuclear contents. Nuclear fractions were run on 10% SDS-PAGE, transferred to nitrocellulose and analyzed by western blotting. Lane 1, recombinant CRT; lane 2, wash of the intact nuclei; lane 3, purified, intact nuclei; lane 4, Triton X-100 soluble, outer nuclear membrane fraction; lane 5, wash of the Triton X-100 insoluble pellet; lane 6, Triton X-100 insoluble, outer membrane stripped, nuclei. Panel A was stained with rabbit anti-CRT antibodies, panel B was stained with goat anti-CRT antibodies and panel C is stained with human anti-lamin B receptor antibodies (as a marker of the inner nuclear membrane). CRT was detected only in the outer membrane fraction with either of the CRT specific antibodies used. Molecular weight markers indicated are as follows: phosphorylase b (101,000), bovine serum albumin (83,000), ovalbumin (50,600), carbonic anhydrase (35,500), soybean trypsin inhibitor (29,100).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GR binding proteins can be included in the reaction to compete for binding to the GR. The GR-protein interaction results in the GR no longer being able to interact with its radiolabeled GRE and the mobility shift is eliminated.

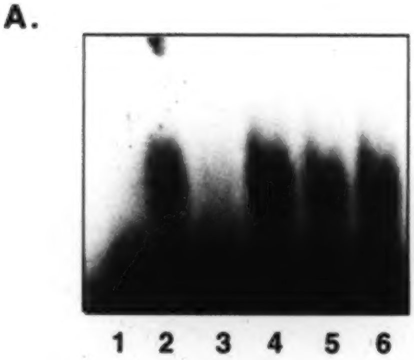
Calreticulin was incubated with GST fused to the DNA binding domain of the GR (GST-GR) and then exposed to ^{32}P labeled GRE (Fig. 4-4A, lane 3). GST-GR was preincubated with its radiolabled GRE followed by addition of calreticulin (Fig. 4-4A, lane 5). As well, calreticulin, GST-GR and radiolabeled GRE were mixed simultaneously (Fig. 4-4A, lane6). All reactions were run on non-denaturing polyacrylamide gel electrophoresis, dried and results visualized by autoradiography. As expected, when GST-GR and calreticulin were allowed to preincubate before the addition of labeled GRE, calreticulin was able to bind to the GR and inhibit its ability to interact with its GRE (Fig. 4-4A, lane 3). When the GR was preincubated with its GRE or when calreticulin, the GR and its GRE were mixed simultaneously calreticulin was no longer able to interact with the GR (Fig. 4-4A, lane 5 and 6). To ensure that this result was not due to a lack of calreticulin being present and therefore being unable to compete for GR binding the DNA mobility shift assay was repeated with the GST-GR preincubated with its labeled GRE followed by the addition of increasing amounts of calreticulin. Fig. 4-4B indicates that even in the presence of a large excess of purified calreticulin the GST-GR-GRE complex (lane 10) could not be disrupted. This indicates that under conditions when the GR is exposed to is GRE calreticulin would not be able to interact with the GR and prevent its binding to its GRE nor would calreticulin be able to displace the already GRE bound GR from the DNA.

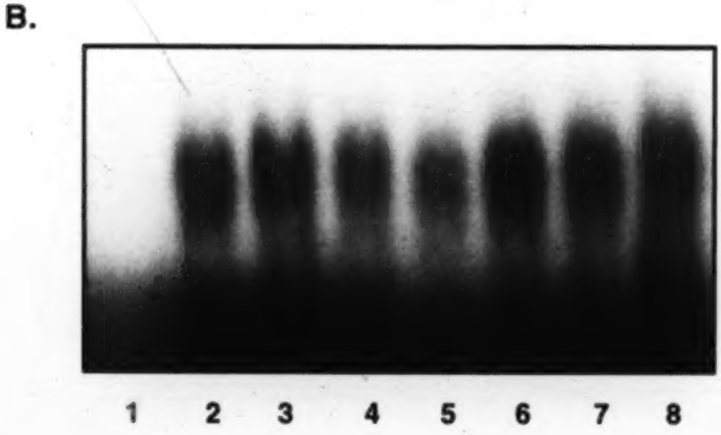
Analysis of calreticulin-GR interaction using the yeast two hybrid system

The previous in vitro studies showed that calreticulin and specifically the N-domain of calreticulin interacted with the DNA binding domain of the GR (Burns *et al.*, 1994). To

Figure 4-4: DNA mobility shift analysis

The ability of dog pancreatic CRT (DP CRT) to bind to the DNA binding domain of the GR fused to GST (GST-GR) was analyzed using the DNA mobility shift assay. Reactions were performed in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM NaCl, 0.05%BSA, 4 mM DTT, 10% glycerol and 2 μg poly dI:dC. **(A)** Lane 1, radiolabeled GRE alone; lane 2, radiolabeled GRE plus GST-GR; lane 3, GST-GR and DP CRT were incubated prior to the addition of the radiolabeled GRE; lane 4, same as lane 2; lane 5, radiolabeled GRE and GST-GR were preincubated before the addition of DP CRT; lane 2, the radiolabeled GRE, GST-GR and DP CRT were all incubated simultaneously. **(B)** in each reaction, the radiolabeled GRE and GST-GR were preincubated before the addition of increasing amounts of DP CRT. Lane 1, radiolabeled GRE alone; lane 2, 4 μg DP CRT; lane 3, 5 μg DP CRT; lane 4, 6 μg DP CRT; lane 5, 7 μg DP CRT; lane 6, 8 μg DP CRT; lane 7, 9 μg DP CRT; lane 8, 10 μg DP CRT.


A.
1 2 3 4 5 6

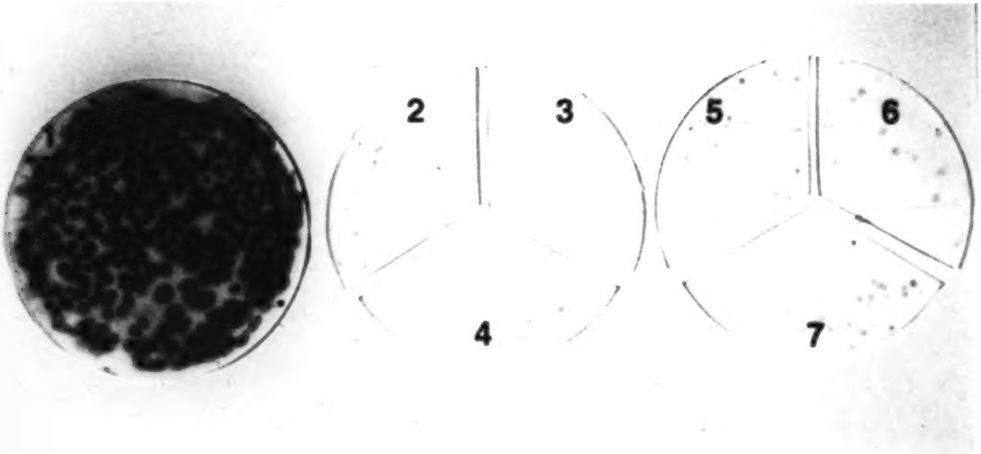

B.
1 2 3 4 5 6 7 8

examine the interaction between calreticulin or the N-domain and the DNA binding domain of the GR the yeast two hybrid system was employed. For this experiment the cDNA encoding the full length mature rabbit calreticulin or the cDNA encoding the N-domain (nucleotides 112-633) were cloned into the yeast expression vector pGBT9 to create pGB-CRT and pGB-N, respectively. These vectors encoded the GAL4 DNA binding domain fused to calreticulin or its N-domain (amino acids 1-174). The cDNA encoding the DNA binding domain of the GR (nucleotides 1389-1650) was cloned into the pGAD424 yeast expression vector to create pGAD-GR. This vector encoded the GAL4 activating domain fused to the DNA binding domain of the GR. The different expression vectors were transformed into yeast in different combinations and their ability to activate the β-galactosidase reporter gene was monitored by the rapid colony lift assay.

pGB-CRT or pGB-N were co-transformed into yeast with the pGAD424 vector as controls to show that calreticulin or the N-domain are not able to activate transcription of the reporter gene when co-expressed with the GAL4 activating domain (Fig. 4-5). Next, pGAD-GR was co-transformed into yeast with the pGBT9 vector to show that the DNA binding domain of the GR does not activate reporter gene expression when co-expressed with the DNA binding domain of GAL4 (Fig. 4-5). Then to examine the potential interaction between the two proteins, pGB-CRT and pGAD-GR and pGB-N and pGAD-GR were co-transformed into yeast. The co-expression of calreticulin and the DNA binding domain of the GR or the N-domain and the DNA binding domain of the GR in the yeast two hybrid system failed to produce any detectable levels of β-galactosidase activity (Fig. 4-5) indicating that the two proteins do not interact *in vivo* under these conditions. As a positive control the pCL1 vector encoding the full length GAL4 protein was transformed into yeast and produced high levels of β-galactosidase activity (Fig. 4-5).

Figure 4-5: Yeast two-hybrid analysis of calreticulin and the GR

Appropriate control vectors or expression vectors encoding CRT or the N-domain fused to the GAL4 DNA binding domain (pGB-CRT and pGB-N, respectively) or encoding the DNA binding domain of the GR fused to the GAL4 activating domain (pGAD-GR) were co-transformed into yeast strain SFY526. The interaction between CRT and the GR was indirectly monitored using the rapid colony lift assay for β-galactosidase activity according to the manufacturer's protocol using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as substrate. Filter 1, yeast was transformed with the pCL1 control vector encoding the full length GAL4 transcription factor; filter 2, yeast was co-transformed with pGAD-GR and pGBT9 (empty vector); filter 3, yeast was co-transformed with pGB-CRT and pGAD424 (empty vector); filter 4, yeast was co-transformed with pGB-CRT and pGAD-GR; filter 5, same as filter 2; filter 6, yeast was co-transformed with pGB-N and pGAD424 (empty vector); filter 7, yeast was co-transformed with pGB-N and pGAD-GR.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7

In order to document that the interaction between calreticulin and other proteins can be monitored by the yeast two hybrid system I examined the interaction between calreticulin and PDI were also being examined. For this study the cDNA encoding full length PDI was cloned into the pGAD424 vector to create pGAD-PDI which encodes for the GAL4 activating domain fused to PDI. The cDNAs encoding for amino acids 1-86 of calreticulin (N1 domain) and encoding for amino acids 169-240 of calreticulin (P1 domain) were cloned into the pGBT9 vector creating pGB-N1 and pGB-P1, respectively.

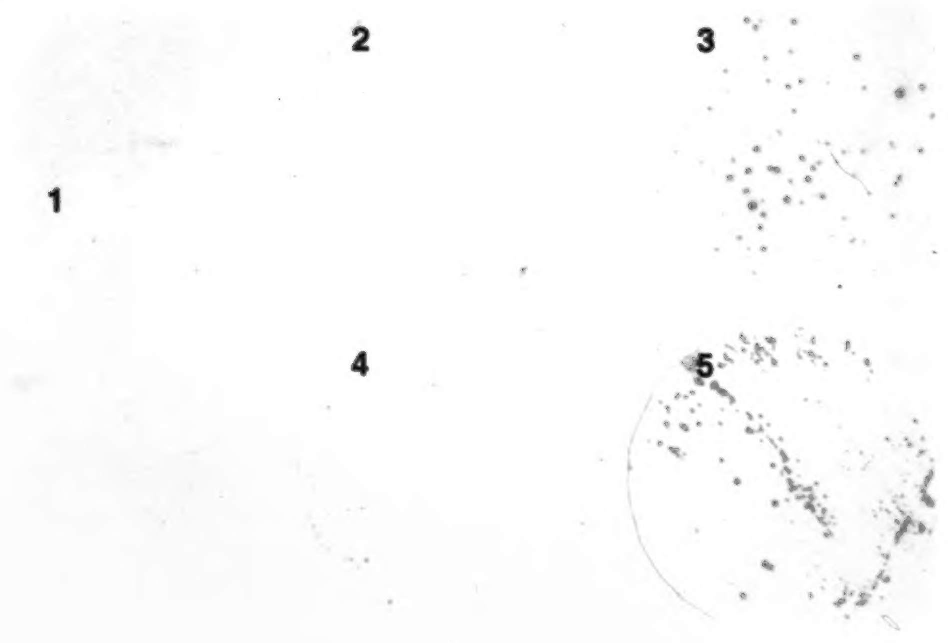
pGAD-PDI was co-transformed with empty pGBT9 and pGB-N1 and pGB-P1 vectors were each co-transformed with pGAD424 as controls. Control experiments did not produce any β-galactosidase activity. When PDI and the N1 domain were jointly expressed, as GAL4 fusion proteins, β-galactosidase expression was induced as detected by the rapid colony lift assay (Fig. 4-6) indicating that PDI and the N1 domain of calreticulin interact *in vivo* under these conditions. β-galactosidase expression was also induced when PDI and the P1 domain of calreticulin were jointly expressed, as GAL4 fusion proteins (Fig. 4-6). Therefore, the yeast two hybrid system for detecting protein-protein interactions can be used successfully when studying calreticulin-protein interactions. This experiment indicates that the GAL4 DNA binding domain fusion proteins with calreticulin or its domains are properly expressed in yeast and they are also properly translocated to the yeast nucleus. This also demonstrates that the lack of an interaction detected between calreticulin and the DNA binding domain of the GR is not due to a lack of protein expression or translocation to the nucleus.

The role of ER or cytoplasmic calreticulin on GR sensitive gene expression

Results of my work showed that calreticulin may not a nuclear protein, and that it does not interact with the GR when the hormone receptor is bound to the GRE in the nucleus. Since calreticulin inhibits steroid sensitive gene expression this leaves the possibility that

Figure 4-6: Yeast two-hybrid analysis of calreticulin and PDI

Appropriate control vectors or expression vectors encoding N1-domain or the P1-domain fused to the GAL4 DNA binding domain (pGB-N1 and pGB-P1, respectively) or encoding full length PDI fused to the GAL4 activating domain (pGAD-PDI) were co-transformed into yeast strain SFY526. The interaction between CRT and the PDI was indirectly monitored using the rapid colony lift assay for β-galactosidase activity according to the manufacturer's protocol using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as substrate. Filter 1, yeast was co-transformed with the pGAD-PDI and pGBT9 (empty vector); filter 2, yeast was co-transformed with pGB-N1 and pGAD424 (empty vector); filter 3, yeast was co-transformed with pGB-P1 and pGAD424 (empty vector); filter 4, yeast was co-transformed with pGB-N1 and pGAD-PDI; filter 5, yeast was co-transformed with pGB-P1 and pGAD-PDI.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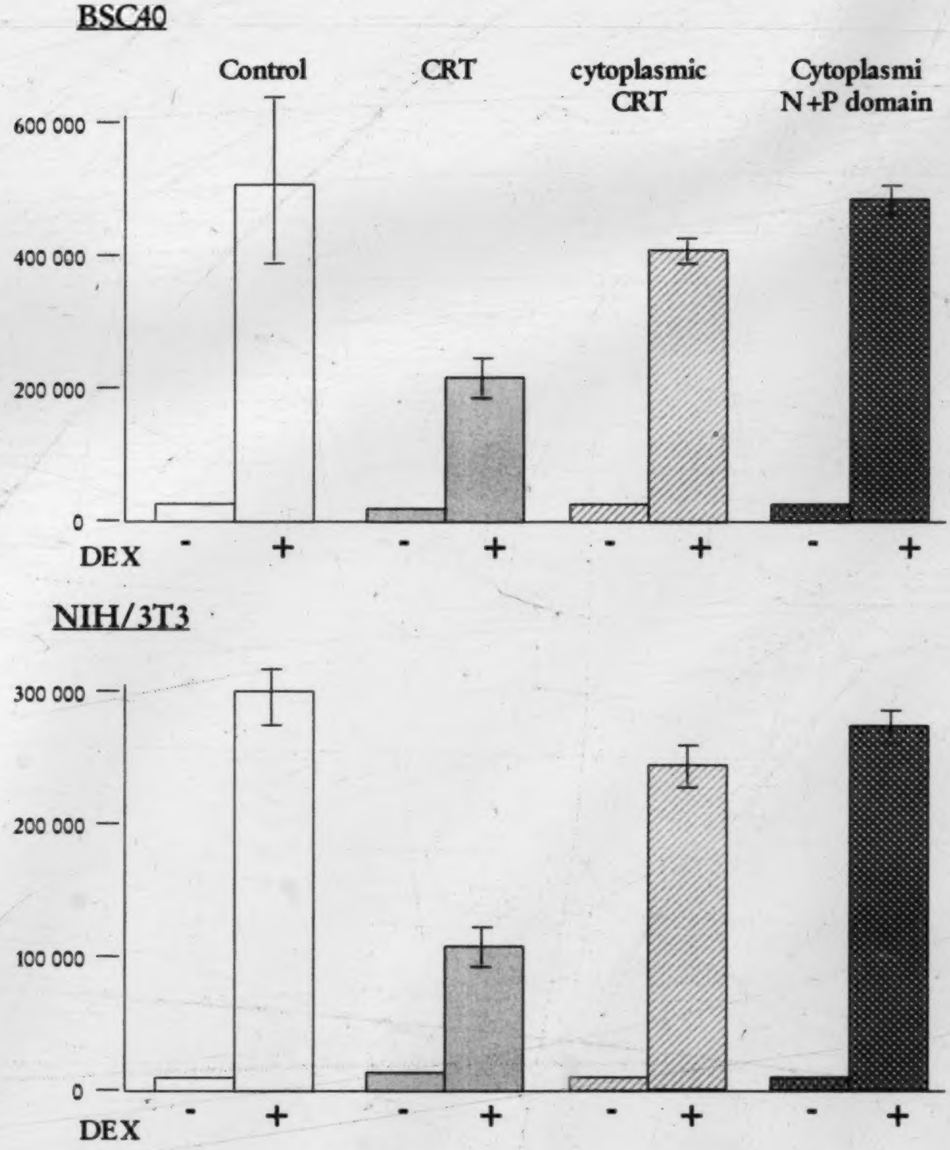
calreticulin may carry out this function from the ER lumen or it is somehow gaining access to the cytoplasm where it interacts with the GR. To address this possibility Kim Burns generated expression vectors encoding calreticulin targeted to the ER, calreticulin without its leader peptide therefore targeting it to the cytoplasm or the N + P domain also lacking its leader peptide targeting it to the cytoplasm creating pSCR-DT, pSCR-L-DT and pSCR-NP, respectively. Each of these vectors included a dystrophin tag that allowed for immunolocalization of the exogenous proteins.

BSC40 and NIH/3T3 cells were co-transfected with the different calreticulin expression vectors, MMTV-GRE-luciferase vector and pβGAL control vector. BSC40 cells lack endogenous GR and were therefore also co-transfected with the VERO plasmid encoding the full-length GR. All results were normalized for transfection efficiency with the pβGAL, β-galactosidase expression vector. Steroid sensitive gene expression was monitored in response to dexamethasone treatment by determining the luciferase activity generated by the MMTV-GRE-luciferase reporter gene construct.

In the presence of 10^{-6} M dexamethasone (Dex), luciferase activity was found to increase >20 fold in either cell type transfected with the MMTV-GRE-luciferase plasmid (or co-transfected with the VERO plasmid for BSC40 cells) over cells not treated with Dex (Fig. 4-7). Co-expression of calreticulin targeted to the ER (pCR-DT) resulted in dramatic decrease is reporter gene activity in the presence of Dex in either cell type indicating that calreticulin is able to inhibit GR sensitive gene expression *in vivo* (Fig. 4-7). Co-expression with calreticulin minus its leader peptide resulting cytoplasmic calreticulin surprisingly did not significantly affect GR sensitive reporter gene activity (Fig. 4-7). Similarly, co-expression with cytoplasmic N+P domain of calreticulin did not have a significant affect (Fig. 4-7). Expression and expected localization of the various exogenously expressed calreticulins were

Figure 4-7: Calreticulin's inhibition of glucocorticoid -sensitive gene expression

BSC40 or NIH/3T3 cells were co-transfected with MMTV-GRE-luciferase vector, pβGAL control vector and different CRT expression vectors as indicated. IN THE CASE OF BSC40, cells the VERO plasmid encoding for the GR was also included in the transfection. Cells were incubated in DMEM containing 10% charcoal treated calf serum for 12 hours followed by incubation for 24 hours with DMEM alone (DEX -) or in DMEM containing 10^{-6} M dexamethasone (DEX +). Cellular extracts were prepared in 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, 50 mM Tris-HCl pH 7.4 and SL inhibitors (described in Chapter 2), and assayed for both luciferase activity as well as β-galactosidase activity. The values shown are relative activities from four independent transfections done in triplicate and normalized for β-galacotsidase activity. Error bars representing ± S.D. are shown.


BSC40
Control
CRT
cytoplasmic CRT
Cytoplasmi N+P domain
600 000
400 000
200 000
0
DEX
-
+
-
+
-
+
-
+
NIH/3T3
300 000
200 000
100 000
0
DEX
-
+
-
+
-
+
-
+

confirmed by immunocytochemistry. This then clearly indicates that it is the ER targeted of calreticulin that of modulates GR sensitive gene expression.

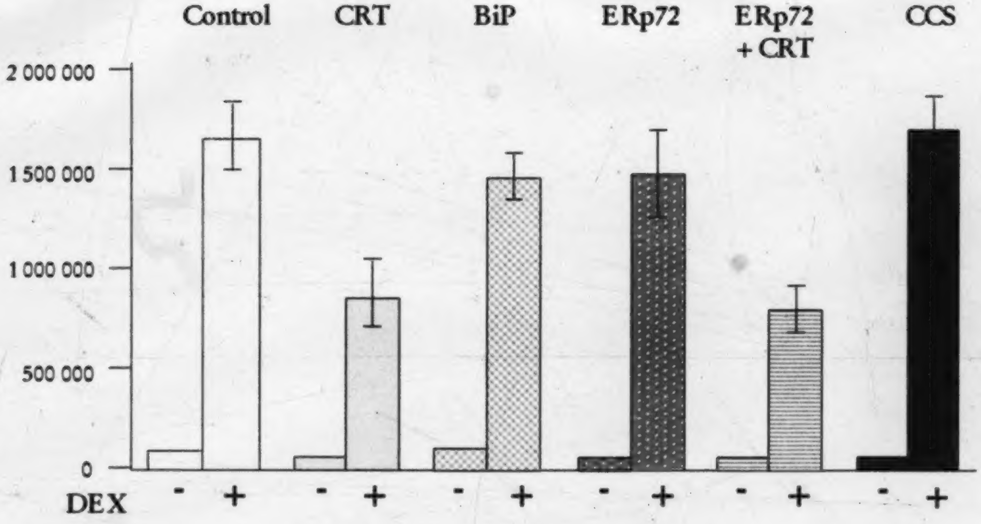
To alleviate any concerns that the affect of overexpressing calreticulin in the ER was due to an overload of protein in the ER lumen the affects of BiP, ERp72 and calsequestrin on GR sensitive reporter gene activity were also examined. BSC40 cells were again co-transfected with the MMTV-GRE-luciferase plasmid, the VERO plasmid, pβGAL and either a plasmid encoding BiP, ERp72 or calsequestrin. BiP and ERp72 are both resident ER protein whereas CCS is located in the SR and has similar physiochemical properties to calreticulin. Fig. 4-8 shows that neither BiP, ERp72 nor CCS had any effect on the detected luciferase activity in the presence of Dex indicating that they do not modulate GR sensitive reporter gene activity. When calreticulin and ERp72 are co-expressed in these cells there is an approximately 50% inhibition of luciferase activity (Fig. 4-8). This level of inhibition is the same as detected when calreticulin is used alone therefore ERp72 does not appear to enhance or detract form calreticulin's effect on GR sensitive reporter gene activity. Inhibition of the GR sensitive reporter gene activity is therefore specific to calreticulin and not a more general affect due to the overexpression of an exogenous protein in the ER lumen.

DISCUSSION

The most important finding of this study is that calreticulin is not a resident nuclear protein and that the protein does not interact with the GR under the *invivo* conditions of the yeast two hybrid system. By immunostaining isolated nuclei with two different antibodies calreticulin was detected in the nucleus with one antibody and not the other. This would indicate that the staining detected with the rabbit anti-calreticulin

Figure 4-8: Calreticulin's inhibition of glucocorticoid-sensitive gene expression is specific to calreticulin

BSC40 cells were co-transfected with MMTV-GRE-luciferase vector, pβGAL control vector, the VERO plasmid encoding for the GR and different expression vectors encoding for ER lumenal proteins as indicated. Cells were incubated in DMEM containing 10% charcoal treated calf serum for 12 hours followed by incubation for 24 hours with DMEM alone (DEX -) or in DMEM containing 10^{-6} M dexamethasone (DEX +). Cellular extracts were prepared in 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, 50 mM Tris-HCl pH 7.4 and SL inhibitors (described in Chapter 2), and assayed for both luciferase activity as well as β-galactosidase activity. The values shown are relative activities from three independent transfections done in triplicate and normalized for β-galacotsidase activity. Error bars representing ± S.D. are shown.



Control
CRT
BiP
ERp72
ERp72
+ CRT
CCS
2 000 000
1 500 000
1 000 000
500 000
0
DEX
-
+
-
+
-
+
-
+
-
+
-
+

antibodies is an artifact or individual isolated nuclei have varying levels of contaminating outer nuclear membrane. Fractionation of the nuclei clearly demonstrated that calreticulin resides in the soluble, outer nuclear membrane fraction only. Since the outer nuclear membrane is continuous with the ER, the calreticulin detected is likely of ER origin. Calreticulin was not found in the insoluble fraction which contains the intact inner nuclear membrane and nuclear contents. Calreticulin does not appear to be localized to the nucleus according to these results.

Calreticulin was unable to interact with the DNA binding domain of the GR when the GR was allowed to interact with its GRE prior to calreticulin addition. As well, calreticulin was unable to inhibit GR binding to its GRE when the three components were introduced to each other simultaneously. Nor could calreticulin's ability to bind to the DNA binding domain of the GR be titrated with increasing amounts of calreticulin. These results show that even if calreticulin could gain access to the nucleus the GR has a much higher affinity for binding to its cognate GRE than to calreticulin and calreticulin would be ineffective as an inhibitor of GR sensitive gene expression. The inability to show that calreticulin is located in the nucleus and calreticulin's inability to bind the GR under what might be considered nuclear conditions led to the conclusion that the direct interaction between calreticulin and the GR may not be physiologically relevant. In support of this conclusion a previous study showed that calreticulin was able to interact *in vitro* with the peroxisome proliferator-activated receptor/retinoid X receptor heterodimers yet was unable to affect transcriptional activation under the control of the heterodimer *in vivo* (Winrow *et al.*, 1995). This indicated that the interaction detected between calreticulin and the hormone receptor heterodimer may occur *in vivo*. These findings are in complete opposition to the accepted belief that calreticulin directly interacted with steroid receptors to modulate their function and suggest that calreticulin may modulate steroid sensitive gene expression from the ER lumen.

Calreticulin's ability to bind to the DNA binding domain of the GR was also examined *in vivo* using the yeast two hybrid system. Calreticulin and the DNA binding domain of the GR were expressed as fusion proteins to the DNA binding domain and activating domain of GAL4, respectively, in yeast. It was found that the two proteins did not interact. There was no detectable reporter gene expression indicative of protein-protein interactions occurring in the yeast nucleus. There is a concern that the yeast two hybrid system may not be suitable for studying calreticulin-protein interactions due to technical difficulties. To work successfully both proteins of interest must be expressed and translocated to the yeast nucleus where if they interact the two portions of the GAL4 transcription factor are brought into close enough proximity to activate gene expression of a reporter gene under GAL4 control. As a positive control for this study the results of calreticulin interacting with PDI in the yeast two-hybrid system were included. These results indicate that calreticulin and PDI are both properly expressed and translocated to the nucleus where they interact and activate reporter gene expression. Therefore, the yeast two hybrid system is a suitable system for studying calreticulin-protein interactions and the negative results obtained with the DNA binding domain of the GR are likely valid.

Calreticulin's ability to inhibit GR sensitive gene expression is well established (Burns *et al.*, 1994) and verified by this study. The effects of expressing calreticulin as a cytoplasmic protein or as an ER protein on GR sensitive reporter gene expression was examined. It was anticipated that calreticulin targeted to the cytoplasm would be a more effective inhibitor of GR sensitive gene expression due to the fact that the GR is located in the cytoplasm allowing direct interaction between the molecules. This was in fact not the case.

Calreticulin, localized to the ER was an effective inhibitor of GR sensitive reporter gene activity whereas cytoplasmic calreticulin had no measurable effect. It was also anticipated that the N+P domain of calreticulin, targeted to the cytoplasm would be a potent inhibitor of GR sensitive gene expression due to the fact that the N-domain, specifically, was found to bind to the DNA binding domain of the GR *in vitro* (Burns *et al.*, 1994). Again, no measurable effect was detected. It is clear that the inhibitory affect of calreticulin on GR sensitive reporter gene activity was specific to calreticulin. Resident ER proteins, BiP and ERp72, had no effect on GR sensitive reporter gene activity and neither did CCS, a calcium binding protein with similar physiochemical properties to calreticulin.

All of these results indicate that calreticulin does not bind to the GR *invivo* but is able to modulate GR sensitive gene expression from the ER lumen. Importantly, this study then changes the focus of calreticulin as a direct regulator of gene expression to calreticulin as an indirect player in the signaling pathway affecting steroid sensitive gene expression. In support of this conclusion is the fact that the previous studies showing calreticulin's ability to inhibit steroid sensitive gene expression were all done with ER localized calreticulin (Burns *et al.*, 1994; Dedhar *et al.*, 1994; Wheeler *et al.*, 1995; Desai *et al.*, 1996). Therefore, direct interaction between calreticulin and the GR is not likely to occur in a physiological setting. For calreticulin to affect steroid sensitive gene expression from the ER a signal must be sent from the lumen of the ER to the nucleus. A plausible conclusion from this study is that calreticulin is indirectly modulating steroid sensitive gene expression by acting as a signaling molecule from the ER.

How can calreticulin function from the ER lumen to affect steroid sensitive gene expression

For the longest time very little was known about "ER signaling" in mammalian cells. One such signaling pathway is the unfolded protein response (UPR) pathway. The UPR pathway responds to an accumulation of malfolded proteins in the ER (Kozutsumi *et al*, 1988) by the induction of expression of resident ER proteins that are known to assist with protein folding. These proteins include BiP (also known as GRP78), GRP94, PDI and ERp72. There has been more extensive data obtained on this pathway in yeast (McMillan *et al.*, 1994). In yeast an ER, transmembrane, serine/threonine kinase/endoribonuclease called Ire1p has been identified as a proximal sensor for the status of unfolded proteins in the ER (Cox *et al.*, 1993; Mori *et al.*, 1993). This kinase is essential for transmitting the signal from the ER to the nucleus to induce gene transcription (Mori *et al.*, 1993; Sham and Walter, 1996). In the recent past evidence has been gathered indicating that there are "ER signaling" pathways functioning in mammalian cells (for review see Pahl and Baeuerle, 1997). The intermediary players involved in the UPR in mammalian cells that sense the accumulation of malfolded proteins and then send the signal to the nucleus to induce gene expression are not yet completely clear. Very recently the human homologue of Ire1p, hIre1p, has been cloned and functionally characterized (Tirasophon *et al.*, 1998). hIre1p contains both the serine/threonine kinase activity and the endoribonuclease activity (Tirasophon *et al.*, 1998) like its yeast counterpart (Cox *et al.*, 1993; Mori *et al.*, 1993) and is capable of modulating reporter gene activity under the control of the BiP promoter (Tirasophon *et al.*, 1998). Although the downstream targets of hIre1p have not been identified yet, the discovery of the UPR signaling molecule indicates the importance of ER to nucleus signaling in mammals.

Another signaling pathway from the ER to the nucleus has recently been described. It is known as the ER-overload pathway (Pahl and Baeuerle, 1997). This pathway involves the activation of an inducible transcription factor known as NF-κB (Pahl and Baeuerle, 1995, Pahl *et al.*, 1996). Generally, NF-κB is found in the cytoplasm bound to its inhibitory subunit IκB which maintains NF-κB in an inactive state (Baeuerle and Baltimore, 1988). Upon a stimulus indicating that there is an overload of proteins in the ER, such as a viral infection, NF-κB is released from its inhibitory subunit, translocates to the nucleus and activates transcription of target genes (for review see Baeuerle and Henkel, 1994). The signal released from the ER that reaches and activates NF-κB is not known. There is evidence that the release of calcium from the ER and subsequent generation of reactive oxygen intermediates (ROIs) are involved in this ER signaling pathway (Pahl et al, 1996; Pahl and Baeuerle, 1996).

Cholesterol biosynthesis and fatty acid biosynthesis are also mediated by another ER-nucleus signaling pathway that involves the sterol regulatory element binding proteins (SREBPs) (Wang *et al.*, 1994; Kim and Spiegelman, 1996; Shimano *et al.*, 1996; Brown and Goldstein, 1997). These SREBPs are ER membrane bound transcription factors that undergo proteolytic processing upon sterol depletion (Sato *et al.*, 1994; Wang *et al.*, 1994). The SREBPs are cleaved by two successive proteases that result in the release of the N-terminal portion of the molecule (Sakai *et al.*, 1996) that is translocated to the nucleus which then activates transcription of genes containing the sterol regulatory element (SRE). Recently the protein responsible for sensing the changes in intracellular sterol levels has been identified. It is known as the SREBP cleavage activating protein (SCAP) (Hua *et al*, 1996). SCAP interacts with SREBP upon sterol depletion and this interaction is required for the initial cleavage of SREBP (Sakai *et al.*, 1997). Whether or not SCAP contains protease activity has not been determined as of yet but it is essential for the proteolytic activation of SREBPs (Sakai *et al.*, 1997). This unique ER-nucleus signaling pathway provides a mechanism for sensing changes in membrane structure and transducing that signal to the nucleus by proteolytic activation of a transcription factor.

The data in this study clearly indicates that it is an ER localized calreticulin that is able to inhibit GR sensitive gene expression With the evidence of ER-to-nucleus signaling pathways mounting it is distinctly possibly that calreticulin is involved in a signaling pathway from the ER. Calreticulin-protein interaction may prove to be essential to calreticulin's ability to affect steroid sensitive gene expression from the ER. Calreticulin has been implicated in other functions around the cell such as modulating integrin function and cell adhesion (Rojiani *et al.*, 1991; Leung-Hagesteijn *et al.*, 1994; Coppolino *et al.*, 1995). For example, overexpression of ER calreticulin results in an increase in vinculin expression and subsequent cell adhesiveness (Opas *et al.*, 1996). It will be important to determine if it is the ER calreticulin that affects integrin function as well.

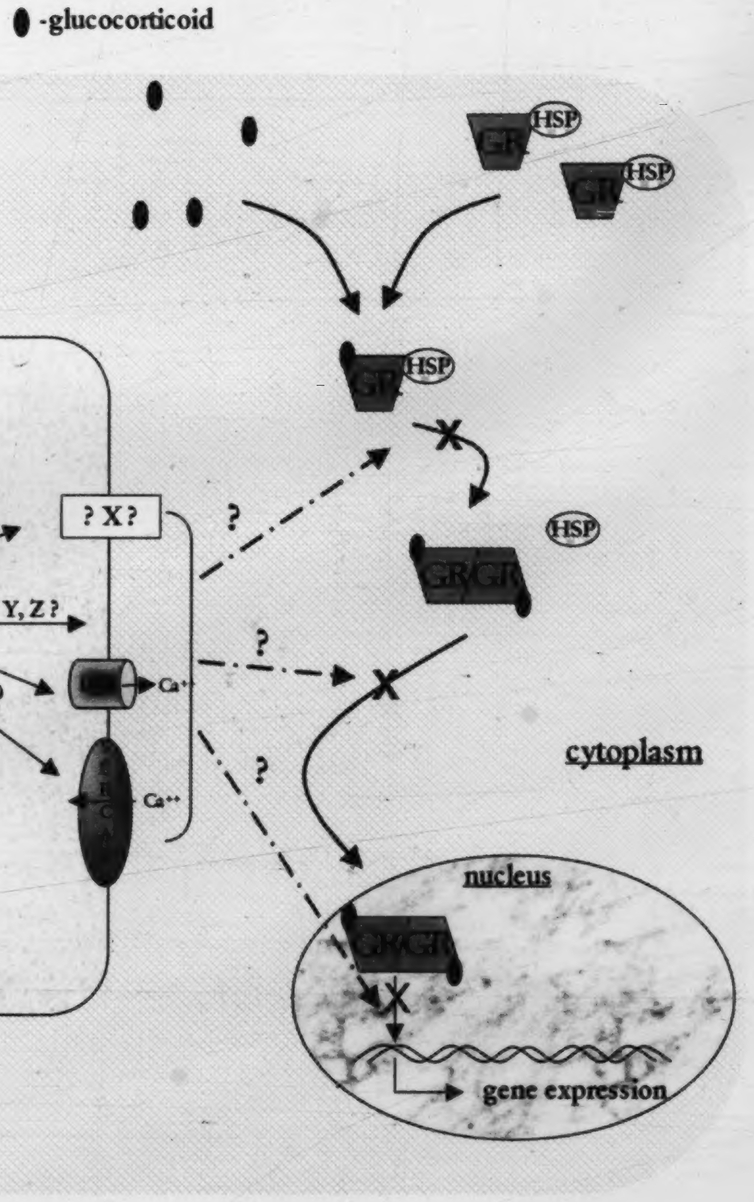
There is the possibility that a "calreticulin receptor" exists located in the ER membrane. Binding to this receptor could start a signaling cascade that would affect steroid sensitive gene expression, along with integrin function or cell adhesion. Another distinct possibility is that calreticulin is affecting cellular functions from the ER by regulating changes intracellular calcium. Calreticulin is well established as a calcium binding protein and over-expression of the protein results in increases in the calcium content of the ER lumenal stores (Liu *et al.*, 1994; Bastianutto *et al.*, 1995, Mery *et al.*, 1996). Calreticulin also has the potential to alter calcium levels in the cytoplasm (Camacho and Lechleiter, 1995). It has already been indicated in the case of NF-κB signaling that calcium release from the ER may be involved in this ER-nucleus signaling pathway (Pahl *et al.*, 1996; Pahl and Baeuerle, 1996). Therefore, it is also possible that calreticulin dependent calcium fluctuations may be involved in "calreticulin signaling" from the ER.

SUMMARY

In this study it was determined that calreticulin is not a resident nuclear protein. The data presented here also established the fact that a direct interaction between calreticulin and the GR receptor is likely of physiological relevance as determined by both the DNA mobility shift analysis and yeast two-hybrid system. These two facts fundamentally change the perspective of calreticulin as a direct modulator of steroid sensitive gene expression. It was also clearly established that calreticulin's ability to inhibit steroid sensitive gene expression is accomplished from its ER location. Cytoplasmic calreticulin does not alter steroid sensitive gene expression as seen in cell transfection experiments that monitored the affect of cytoplasmic calreticulin on reporter gene activity under the control of a GR sensitive promoter. This data opens up the possibility that calreticulin is somehow involved in a signaling pathway from the ER to the nucleus (Fig. 4-9). This study will likely have profound affects on the direction of calreticulin research in the near future.

Figure 4-9: Putative model of calreticulin signaling from the endoplasmic reticulum to the nucleus

This is a schematic diagram outlining possible signaling pathways that may explain CRT's ability to inhibit glucocorticoid-sensitive gene expression from the ER. The potential existence of a CRT receptor is depicted by the white rectangle labeled **X**. Other ER lumenal proteins that may be involved are shown as **Y** and **Z**. The GR exists as a monomer bound by HSPs in the cytoplasm until the glucocorticoid hormone binds to the GR. The HSPs then dissociates, the GR homodimerizes and translocates to the nucleus where it activates gene transcription of genes containing the GRE. It is unknown at what stage CRT may be affecting the GR which is depicted by the broken arrows labeled with question marks.


-glucocorticoid
GR
HSP
GR
HSP
GR
HSP
ER
lumen
? X ?
?
CRT
Ca++
Ca++
Ca++
Ca++
? Y, Z ?
?
Ca++
?
Ca++
HSP
GR/GR
cytoplasm
Ca++
?
CRT
PDI
nucleus
GR/GR
gene expression

CHAPTER FIVE

CALCIUM DEPENDENT INTERACTION BETWEEN CALRETICULIN AND PERFORIN

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INTRODUCTION

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are essential components of the cell-mediated arm of the immune system. They are absolutely required for the effective destruction of virus infected and tumorigenic cells (Berke, 1995; Liu *et al.*, 1995; Podack, 1995; Kägi and Hengartner, 1996). One method of CTL killing of target cells relies on the cytolytic granules (Atkinson and Bleackley, 1995; Lowin *et al.*, 1995). These cytolytic granules carry effector molecules, perforin, granzymes and proteoglycans (reviewed in Lowin *et al.*, 1995; Redmond *et al.*, 1987; Tschopp and Jongeneel, 1988; Stevens *et al.*, 1989; Tschopp and Nabholz, 1990; Griffiths and Argon, 1995; Griffiths, 1997) that are part of the killing machinery. Calreticulin is also a component of the CTL granules (Dupuis *et al*, 1993). It was found that calreticulin expression is upregulated in stimulated T-cells (Burns et al., 1992) and later shown that not only is calreticulin a component of the CTL granules but also that calreticulin co-purifies with perforin (Dupuis *et al.*, 1993). This led to the following study where the potential interaction between calreticulin and perforin was examined.

Perforin is part of the lethal hit delivered by activated CTLs to a target cell during the killing process. Perforin is sequestered in the cytolytic granules in an inactive state and upon release from the granules it inserts into the membrane, polymerizes and forms a pore through the target cell membrane (reviewed in Lowin *et al.*, 1995). Perforin's ability to bind to phospholipids and polymerize into pore structure both require calcium (Blumenthal *et al.*, 1984; Henkart *et al.*, 1984; Podack *et al.*, 1985; Masson *et al.*, 1990) It has been determined that perforin is essential for the efficient T-cell mediated killing of virus infected cells (Kägi *et al.*, 1994; Lowin *et al.*, 1994). Perforin is able to inflict cell death simply by causing osmotic lysis or more commonly by supplying the required elements to activate apoptosis (Zychlinsky *et al.*, 1991; Podack, 1995). The apoptotic pathway is initiated after granzyme B gains access to the target cell resulting the cleavage and activation of Ced-3-like proteases (Froelich *et al.*, 1996). Specifically, the cysteine protease caspase 3 (CPP32, Yama, apopain) was found to be activated and responsible for initiating the DNA fragmentation of the apoptotic process (Darmon *et al.*, 1995; Darmon *et al.*, 1996).

The predicted amino acid sequence of perforin from its cDNA sequence indicates that it is a 534 amino acid protein after the removal of a 21 amino acid signal peptide that targets it to the ER (Lichtenheld *et al.*, 1988). Perforin contains two potential N-linked glycosylation sites and a 300 amino acid stretch in the middle of the molecule that is thought to be responsible for pore formation due to its homology with C9 of the complement proteins (Lichtenheld *et al.*, 1988; Lowrey et al., 1989). The N- and C- terminal regions do not show any homology with other proteins and their functions are not known.

Perforin efficiently kills target cells but the T-cell manages to synthesize perforin without any harm to itself. The granules where perforin is stored in the cell contain proteoglycans (reviewed in Griffiths and Argon, 1995) and have a very low pH. The granules contain a proton pump that maintains the pH at approximately 5.5 (Kataoko et al., 1994; Kataoka et al., 1996). This acidic environment is thought to favour interactions between perforin and the proteoglycans which would then assist in maintaining perforin in an inactive state (Masson *et al.*, 1990). Upon exocytosis of the granule contents the pH increases, the proteoglycans would then dissociate and exposure to anything greater than 100 μM calcium causes the transition to the activated state and perforin becomes lethal (Tschopp and Nabholz, 1990; Young *et al.*, 1987). When perforin is initially synthesized it passes through the ER which has relatively neutral pH (Mellman *et al.*, 1986) and been estimated to have at least 200 μM free calcium (Montero *et al.*, 1995). This environment would cause perforin to become activated. Instead perforin is synthesized as an inactive precursor as to protect the cell as it passes through the ER (Uellner *et al.*, 1998). Perforin is cleaved during its biosynthesis once it reaches an acidic compartment (presumably the cytolytic granules) (Uellner *et al.*, 1998). This cleavage results in the exposure of a phospholipid-binding domain which then gives perforin the potential to become activated (Uellner *et al.*, 1998) but due to the proteoglycans, and maybe calreticulin, it is held in an inactive state until the CTL responds to a target cell and secretes the granule contents.

Calcium is an essential second messenger in T-cell activation (Weiss and Imboden, 1987; Premack and Gardner, 1992; Crabtree and Clipsotne, 1994). T-cell activation results in an increase in intracellular calcium levels in both ER and non-ER calcium stores (Clementi *et al.*, 1994). The release of calcium is involved in the activation of several transcription factors resulting in the increased expression of the appropriate T-cell activation associated genes (Rothenberg and Ward, 1996). There is also an increase in expression of several ER proteins. It was established that calreticulin expression is elevated in activated T-cells (Burns *et al.*, 1992) and later discovered that PDI and Serca 2b expression were also increased in response to T-cell activation (Clementi *et al.*, 1994). Interestingly, the elevated calcium levels were found to be associated with a thapsigargin sensitive, IP_3 insensitive store indicating the involvement of Serca 2B in a region of the ER possibly seperated from the IP_3 receptor (Clementi *et al.*, 1994). The increased Serca 2B expression was found to remain localized with the ER (Launay *et al.*, 1997) whereas calreticulin was found in the CTL granules (Dupuis *et al.*, 1993). The localization of PDI upon T-cell activation has not been explored.

The events surrounding CTL activation and cell-mediated killing of target cells are obviously very complex. Understanding the players involved in the process is then essential to delineating the mechanisms involved. Calreticulin's localization to the CTL granules (Dupuis *et al.*, 1993) is both intriguing and puzzling. It is not known how calreticulin might escape the KDEL retention/retrieval mechanism of the ER. Nor is it understood what calreticulin might be doing in the CTL granules. Calreticulin's co-localization and co-purification with perforin led to the belief that calreticulin may be interacting with perforin. This study was designed to address that possibility. The potential interaction between calreticulin and perforin was examined both *in vitro* and *invivo*.

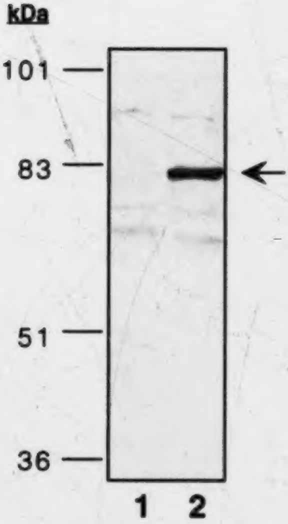
RESULTS

Generation of anti-calreticulin antibodies specific for the KDEL amino acid sequence

Specific antibodies to the amino acid sequence Q-A-K-D-E-L representing the last six amino acids of calreticulin were generated to address the question of whether or not CTL granule calreticulin contains its KDEL ER retention/retrieval sequence. Rabbits were immunized with the keyhole limpet hemocyanin conjugated Q-A-K-D-E-L peptide three times at regular intervals and the resulting serum harvested. The antibodies produced, designated CRT283, were enriched by affinity purification against a BSA conjugated Q-A-K-D-E-L peptide affinity column. The specificity of the antibodies was verified by Western blot. *E. coli.* lysates containing truncated GST-calreticulin fusion protein lacking the C-terminal KDEL amino acid sequence or full length GST-calreticulin fusion protein were run on SDS-PAGE, transferred to nitrocellulose and probed with the affinity purified CRT283 antibodies. Figure 5-1 shows that CRT283 antibodies clearly recognize full length GST-calreticulin fusion protein (lane 2) but did not recognize the truncated fusion protein lacking

Figure 5-1: Sensitivity of CRT283 antibodies

CRT283 antibodies used are specific for the KDEL amino acid sequence of CRT. *E.coli* lysates containing GST-CRT fusion proteins were run on 10% SDS-PAGE, transferred to nitrocellulose and probed with the CRT283 antibodies (dilution 1:100). Lane 1, truncated recombinant CRT lacking the C-terminal KDEL ER retention/retrieval amino acid sequence fused to GST; lane 2, full length, mature recombinant CRT fused to GST. The arrow indicates the location of the GST-CRT fusion protein. Molecular weight markers indicated are as follows: phosphorylase b (101,000), bovine serum albumin (83.000), ovalbumin (50,600), carbonic anhydrase (35,500).


kDa
101
83
51
36
1
2

the KDEL amino acid sequence (lane 1). The CRT283 antibodies are therefore specific for calreticulin's C-terminal KDEL retention sequence.

Calreticulin present in cytolytic granules contains its KDEL sequence

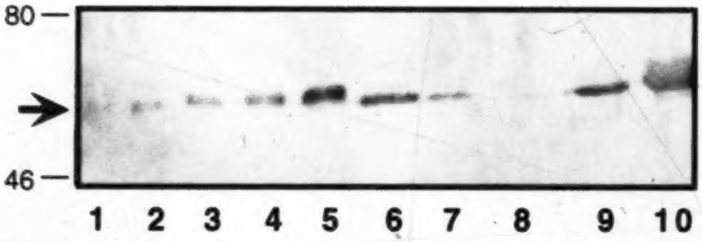
To determine whether or not calreticulin is present in the cytolytic granules contains its KDEL retention sequence western blotting was performed with the CRT283 antibodies. Increasing amounts of purified calreticulin was analyzed to show the high affinity of the CRT283 antibodies for calreticulin (Fig. 5-2, lanes 1-5, 10). Calreticulin was detected in extract from unfractionated, stimulated cytolytic granules as well as in the purified perforin preparation from stimulated cytolytic granules. This verifies that calreticulin is located in the cytolytic granules and that it does co-purify with perforin (Dupuis *et al.*, 1993). Importantly, calreticulin detected in the granules and co-purifying with perforin does have an intact KDEL retention sequence as indicated by the use of the CRT283 antibody. Lysates from resting and stimulated T-cells were also analyzed for the presence of calreticulin (Fig. 5-2, lanes 8 and 9). Increased calreticulin expression is detected in stimulated T-cell lysate (lane 9) compared with in the resting T-cell lysate (lane 8) where calreticulin is barely detectable. This then confirms the finding that calreticulin expression is increased upon T-cell stimulation (Burns *et al.*, 1992). Calreticulin detected in the T-cell lysates also contains its KDEL sequence. Clearly, calreticulin then must circumvent the KDEL retrieval mechanism in the cell or have targeting information that overrides the retention signal upon T-cell stimulation.

Co-purification of calreticulin with perforin but not with granzymes

In order to determine whether calreticulin associates with other proteins in the cytolytic granules, isolated granules were fractionated by metal-affinity chromatography. A Cu^{2+} IMAC column was used to separate proteoglycans, which do not bind to the column, and

Figure 5-2: Presence of KDEL retention signal on calreticulin form cytotoxic T-lymphocytes

The presence of the KDEL retention/retrieval amino acid sequence on CRT from CTL granules was evaluated by western blot using the CRT283, KDEL specific antibodies. Isolated CTL granules or CTL lysates were run on 10% SDS-PAGE, transferred to nitrocellulose and probed with the CRT283 antibodies (dilution 1:100). Lane 1-5 contains increasing amounts of recombinant CRT to indicate the specificity of the CRT283. Lane 1, 10 ng; lane 2, 20 ng; lane 3, 50 ng; lane 4, 100 ng; lane 5, 500 ng; lane 6, unfractionated CTL granule extract; lane 7, Co^{2+}-IMAC isolated perforin; lane 8, lysate from resting CTLs; lane 9, lysate from ConA stimulated CTLs; lane 10, 2 µg dog pancreatic CRT. The arrow indicates the position of calreticulin. Molecular weight markers indicated are as follows: bovine serum albumin (80,000), ovalbumin (46,000).


80
46
1
2
3
4
5
6
7
8
9
10

granzymes away from perforin. Fractions were run on SDS-PAGE and probed with anti-perforin antibodies (Fig. 5-3A) or goat anti-calreticulin antibodies (Fig. 5-3B). Perforin was detected in the unfractionated granule proteins (Fig. 5-3A, lane 2) as well as in the eluted fraction containing purified perforin (lane 3). Calreticulin was found associated with the purified perforin fraction (Fig. 5-3B, lane 3) indicating that calreticulin does co-purify with perforin. Calreticulin was barely detected in the unbound fraction (Fig. 5-3B, lane 4) and not detected at in the granzymes fraction (Fig. 5-3B, lane 5). These fractions were also analyzed for the presence of BiP, PDI, ERp72, Grp96 and Serca 2 but none of these other ER proteins were detected in any of the granules fractions. This indicates that calreticulin does not associate with granzymes in the cytolytic granules but specifically associates with perforin and that this association is specific to calreticulin.

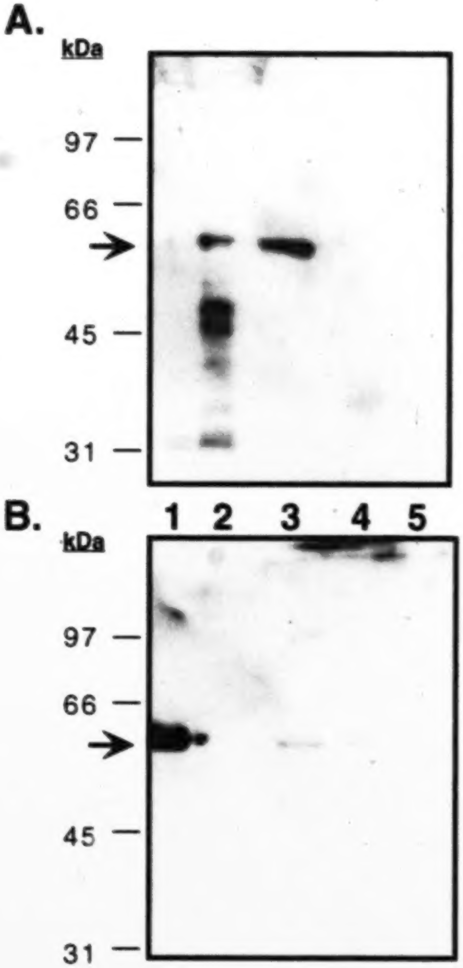
Localization of calreticulin to the cytolytic granules using the green fluorescent protein

To verify that calreticulin is targeted to the cytolytic granules the localization of calreticulin fused to GFP was examined. YT Indy cells were used in this experiment due to the fact that they constitutively produce granules and therefore do not require stimulation. YT Indy cells were transfected with a construct encoding GFP fused to the N-terminus of full length mature calreticulin. The fusion protein was targeted to the ER with the N-terminal signal

sequence from calreticulin cloned directly upstream of the cDNA sequence encoding GFP followed by the cDNA sequence encoding calreticulin and ending with the sequence encoding the KDEL retention signal. Cells were selected for the stable expression of the GFP-calreticulin construct. The cells were examined for fluorescence by confocal

Figure 5-3: Co-purification of calreticulin with perforin

CTL granules were isolated and fractionated by Co^{2+} IMAC chromatography. The column fractions were separated on 10% SDS-PAGE, transferred to nitrocellulose and analyzed by western blot with either (**A**) monoclonal anti-perforin antibodies (dilution 1:100) or (**B**) goat anti-CRT antibodies (dilution 1:300). Lane 1, purified dog pancreatic CRT; lane 2, unfractionated granule proteins; lane 3, perforin-containing fraction; lane 4, column flow through containing the unbound proteoglycans; lane 5, granzyme-containing fraction. The arrows indicate the position of perforin in (**A**) and calreticulin in (**B**). Molecular weight markers indicated are as follows: Molecular weight markers indicated are as follows: phophorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700) and bovine carbonic anhydrase (31,000).


A.
kDa
97
66
45
31
B.
1
2
3
4
5
kDa
97
66
45
31

microscopy in Dr. Opas' lab. Although YT Indy cells are very small and clear resolution was difficult GFP-calreticulin was detected in the transfected cells in what appears to be granular structure (Fig. 5-4). This evidence supports previous data localizing calreticulin to the cytolytic granules (Dupuis et al., 1992) and establishes that calreticulin produced and targeted to the ER does manage to escape the KDEL retention/retrieval mechanisms.

Calreticulin is localized to the granules in perforin knockout mice

One possible explanation for calreticulin localizing to the cytolytic granules is that calreticulin acts as a chaperone for perforin traveling through the ER to the granules. This then could result in calreticulin tagging along with perforin to the granules while helping to maintain perforin in an inactive conformation. To explore this possibility Michael Pinkoski examined the localization of calreticulin in CTLs isolated from wild-type and perforin knock-out mice. The CTLs were attached to poly-L-lysine coated coverslips, fixed, permeablized and immunostained with antibodies to calreticulin or granzyme B. Figure 5-5 demonstrates that calreticulin is detected in the granules of both CTLs isolated from wild type (panel C) and perforin knockout mice (panel A). Staining with anti-granzyme B antibodies verified that the calreticulin detected co-localized with granzyme B in both wild-type (panel D) and perforin knockout mice (panel B) to the cytolytic granules. These results suggest that calreticulin's targeting to the cytolytic granules is independent of perforin's localization. Calreticulin is not likely acting as a chaperone for perforin as perforin travels through the ER to its destination in the granules.

Co-immunoprecipitation of calreticulin and perforin

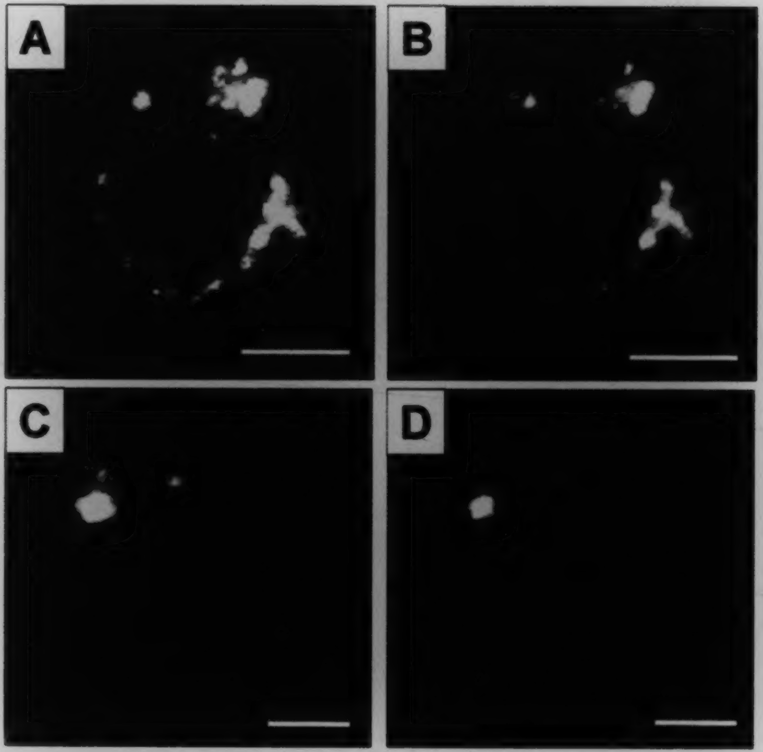
Due to the fact that perforin's activation and killing ability is absolutely calcium dependent (Blumenthal *et al.*, 1984; Henkart *et al.*, 1984; Podack *et al.*, 1985; Masson *et al.*, 1990) the role of calcium was explored in the interaction between calreticulin and perforin.

Figure 5-4: GFP-calreticulin localization in YT-INDY cells

YT-INDY cells, that constitutively produce CTL granules, were transfected with the GFP-CRT expression vector that encodes for full length CRT fused to the GFP. The endogenous CRT signal sequence was included at the 5' end of the cDNA to target the fusion protein to the ER. Cells were selected for the stable incorporation of the GFP-CRT plasmid using 400 μg/mL G418. Stably transfected cells were attached to poly-L-lysine coated coverslips, fixed and mounted in Vinol 205S. The cells were then examined by confocal microscopy (Dr. M. Opas, University of Toronto). GFP-CRT was detected in a pattern indicative of granule localization.

Figure 5-5: Calreticulin localization to perforin knockout CTL granules

Granules from normal (panel C and D) and perforin knockout mice (panel A and B) were examined for CRT localization using immunocytochemistry. CTLs were adhered to glass coverslips, fixed and permeablized followed by indirect immunofluorescence with goat anti-CRT antibodies (panel A and C) or mouse anti-granzyme B antibodies (panel B and D). CRT is found co-localized to the CTL granules with granzyme B regardless of the presence of perforin

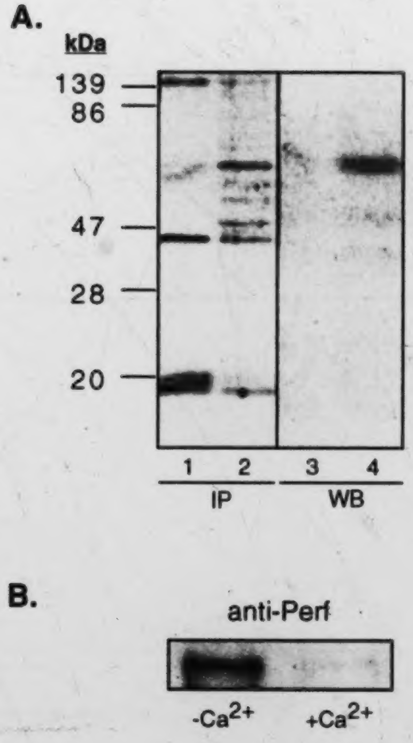

A
B
C
D

The immunoprecipitation experiment was repeated in the presence or absence of calcium to identify any calcium dependence. Solublized rat granules were incubated with anti-calreticulin N1 antibodies specific for the N-terminal domain of calreticulin in the presence of 1.5 mM Ca^{2+} or in the absence of any exogenous calcium. The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by western blotting with monoclonal anti-perforin antibodies. The western blot revealed that perforin was only pulled down with calreticulin in the absence of calcium (Fig. 5-6B). In the presence of calcium perforin was not detected in the calreticulin immunoprecipitate. This confirmed that calreticulin and perforin do interact and this interaction is calcium dependent in that the interaction only occurs in the absence of calcium.

Dr. Tschopp's lab examined the potential interaction between calreticulin and perforin by immunoprecipitation. Extracts form mouse B6.1 T-cells, labeled with [^{35}S]-methionine, were incubated with anti-calreticulin antibodies N1 or with control antibodies. The immunoprecipitates were analyzed by SDS-PAGE and western blotting. The proteins present in the immunoprecipitates, separated by SDS-PAGE were visualized by autoradiography and revealed a prominent band corresponding to a 60kDa protein along with three other less prominent bands ranging from 47kD to approximately 58kDa (likely representing calreticulin) (Fig, 5-6A, lanes 1 and 2). The western blot of the same immunoprecipitates revealed that the 60kDa band detected in the autoradiograph was indeed perforin (Fig. 5-6A, lanes 3 and 4). Perforin was only detected in the immunoprecipitate with the specific anti-calreticulin antibody (lane 4) and not with control serum (lane 3). Under immunoprecipitation conditions, calreticulin interacts with perforin.

Figure 5-6: Co-immunoprecipitation of perforin with calreticulin

The perforin-CRT complex was immunoprecipitated with rabbit anti-CRT N1 antibodies from [^{35}S]-methionine labeled cell extracts from mouse B6.1 T-cell line (**A**) or from purified mouse CTL granules (**B**). Panel **A**: lanes 1 and 2, immunoprecipitated proteins detected by autoradiography; lanes 3 and 4 immunoprecipitated proteins analyzed by western blotting with anti-perforin antibodies (dilution 1:100). Lanes 1 and 3, control serum; lane 2 and 4, immunoprecipitate with anti-CRT N1 antibodies. Panel **B**: immunoprecipitation with anti-CRT N1 antibodies was carried out in the presence or absence of Ca^{2+} as indicated followed by western blot analysis of the immunoprecipitate with monoclonal anti-perforin antibodies (dilution 1:100).


A.
kDa
139
86
47
28
20
1
2
3
4
IP
WB
B.
anti-Perf
-Ca2+
+Ca2+

Perforin interacts specifically with the N- and P- domain of calreticulin

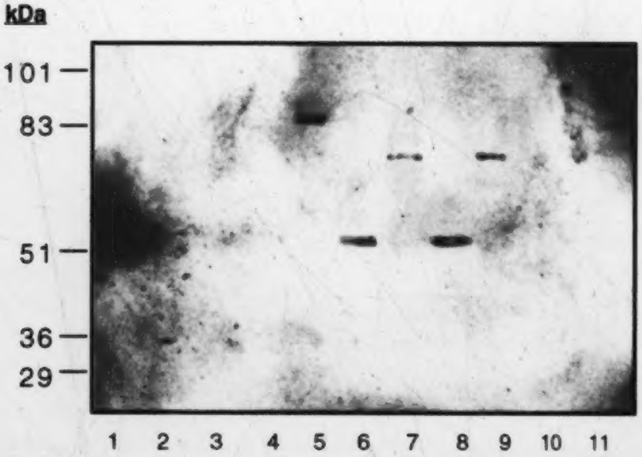
To determine what region of calreticulin is involved in the interaction with perforin the protein overlay technique was employed. Full length, mature, [^{35}S]-methionine labeled perforin was produced in the cell free T7 transcription/translation coupled system (Promega) and used in the protein overlay. Purified GST-calreticulin fusion protein along with GST fused to the various calreticulin domains were separated by SDS-PAGE, transferred to nitrocellulose and probed with [^{35}S]-methionine perforin (Fig. 5-7, lanes 5-10). BSA, CCS, PDI and GST alone were also included on the blot as negative controls. In the absence of calcium, radiolabeled perforin bound to full length calreticulin, the N-domain, the N+P-domain, the P-domain and the P+C-domain (Fig. 5-7, lanes 5,6,7,8 and 9, respectively). Radiolabeled perforin did not bind to the C-domain (Fig. 5-7, lane 10) nor did it bind to BSA, CCS, PDI or GST alone (Fig. 5-7, lanes 2,3,4 and 11, respectively). The protein overlay was also performed in the presence of 0.1 mM Ca^{2+} but no radiolabeled perforin was detected binding to any of the proteins. This confirms the fact that calreticulin and perforin interact in the absence of calcium. It also established that the N- and P-domains of calreticulin are involved in the interaction.

The P-domain of calreticulin interacts with perforin *in vivo*

The yeast two hybrid system was used to determine if calreticulin was capable of interacting with perforin *in vivo*. This system was also used to verify the regions of calreticulin involved in the interaction with perforin in a more physiologically relevant environment. The calreticulin domains were expressed in yeast as fusion proteins with the GAL4 DNA binding domain. The shorter N1-domain encompassed amino acid residues 1-86 while the full length N-domain covered residues 1-174. The P-domain covered residues

Figure 5-7: Protein overlay with ^{35}S labeled-perforin

The protein overlay technique was employed to examine which protion of CRT may be involved in the CRT-perforin interaction. CRT or its domains fused to GST were expressed in E.coli, purified and separated by SDS-PAGE. The proteins were transferred to nitrocellulose and incubated in the absence of Ca^{2+} with ^{35}S labeled-perforin. Bound perforin was visualized by autoradiography. Lane 1, molecular weight markers; lane 2, BSA; lane 3, cardiac calsequestrin; lane 4, PDI; lane 5, GST-CRT; lane 6, GST-N; lane 7, GST-N+P; lane 8, GST-P; lane 9, GST-P+C; lane 10, GST-C; lane 11, GST alone. Molecular weight markers indicated are as follows: Molecular weight markers indicated are as follows: phophorylase b (101,000), bovine serum albumin (83,000), ovalbumin (50,600), bovine carbonic anhydrase (35,500) and soybean trypsin inhibitor (29,100).


kDa
101
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51
36
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1
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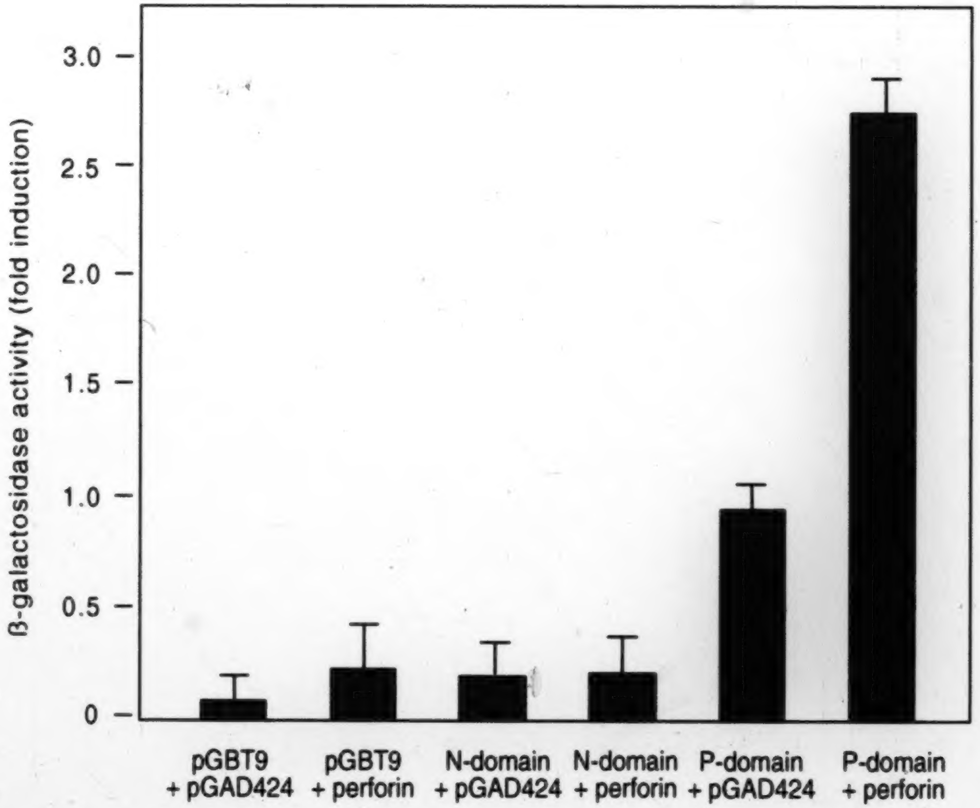
11

169-289 while the shorter P1-domain included residues 169-240. Full length, mature perforin was expressed as a fusion protein with the GAL4 activating domain. The various constructs were transformed into yeast in various combinations and the expression of the β-galactosidase reporter gene monitored as an indication of protein-protein interactions.

The N-, N1-, P- and the P1-domain did not activate the reporter gene expression when expressed in yeast alone nor did they significantly activate the reporter gene expression when co-expressed with the GAL4 activating domain. There was also no detectable reporter gene activity when the perforin fusion protein was expressed alone or co-expressed with the GAL4 DNA binding domain. No reporter gene activity was detected when the perforin fusion protein was co-expressed with the N- nor the N1-domain of calreticulin fused with the GAL4 DNA binding domain. Co-expression of the P1-domain or the P-domain and perforin fusion proteins activated expression of the β-galactosidase reporter gene. The level of the reporter gene activity was quantitated using a liquid β-galactosidase assay. Figure 5-8 shows that when perforin and the P-domain are co-expressed in the yeast two hybrid system there is a 2.5 - 3.0 fold increase in the β-galactosidase activity detected indicating that perforin interacts with the P-domain of calreticulin under these *in vivo* conditions. The average of three experiments is presented and each experiment was done in triplicate. This is in support of the protein overlay experiment which also indicated that the P-domain was involved in the interaction of calreticulin and perforin *invitro*. The reason why there was no interaction detected between perforin and the N-domain of calreticulin in the yeast two hybrid system is not clear. It appears that the interaction with perforin is strongly dependent on the P-domain of calreticulin.

Figure 5-8: Yeast two-hybrid analysis of CRT and perforin

Appropriate control vectors (pGBT9 or pGAD424) or expression vectors were co-transformed into yeast strain SFY526 as indicated. Vectors used encoded for the N-domain or the P-domain fused to the GAL4 DNA binding domain or full length perforin fused to the GAL4 activating domain. The interaction between the CRT domains and perforin was indirectly monitored and quantitated using the liquid assay for β-galactosidase activity according to the manufacturer's protocol using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. Results are shown as fold induction of β-galactosidase activity over control yeast cells and represent two independent experiments performed in triplicate. Error bars indicating the S.D. are shown.


ß-galactosidase activity (fold induction)
3.0
2.5
2.0
1.5
1.0
0.5
0
pGBT9 + pGAD424
pGBT9 + perforin
N-domain + pGAD424
N-domain + perforin
P-domain + pGAD424
P-domain + perforin

DISCUSSION

With the specific antibodies generated in this study it was determined that the calreticulin located in the cytolytic granules, that co-purifies with perforin, does contain its KDEL ER retention signal. It is possible that calreticulin's interaction with perforin somehow masks the KDEL sequence on calreticulin but this is unlikely. Through the protein overlay experiment and the yeast two hybrid experiment it was demonstrated that calreticulin's interaction with perforin is strongly dependent on the P-domain of calreticulin. The KDEL retention sequence of calreticulin actually covers the last four amino acids of the protein. It is unlikely that the interaction between the P-domain and perforin would interfere with the C-terminal KDEL sequence. Also, this study clearly showed that calreticulin is targeted to the granules regardless of the presence of perforin. In perforin knockout mice it was established that calreticulin is still located in the granules of CTLs therefore it is not necessary for perforin to mask the KDEL retention sequence of calreticulin for calreticulin to escape the ER.

Analysis of calreticulin's amino acid sequence reveals two potential lysosome targeting signals. The putative targeting signals are located at amino acid residue 42-48 and 347-353 (Dice, 1990) within the N- and C-domains of the protein, respectively (Michalak *et al.*, 1992). It is possible that within the CTLs the lysosomal targeting signals located within calreticulin override the KDEL retention signal. As the stimulated CTL starts producing large quantities of calreticulin (Burns et al., 1992) the increased concentration of calreticulin may show preference to the lysosomal targeting signal. It is also possible that the elevated calreticulin concentration in the stimulated CTLs simply overloads the KDEL pathway re-routing calreticulin through a secondary pathway resulting in localization to the granules. The theory has been put forth that localization of some proteins to different subcellular compartments, yet encoded by a single gene, may be due to competition between different targeting signals present within the protein (Danpure, 1995). This is also supported by the fact that other proteins in the KDEL family of proteins, such as BiP, PDI, Erp72 and Grp96, were not detected in the granules. All these proteins contain KDEL retention signals but lack lysosomal targeting sequences indicating that calreticulin is the only ER protein known that escapes the ER retention/retrieval mechanisms and localizes to the cytolytic granules. There has been evidence of low levels of Grp96 escaping the ER and becoming localized on the cell surface (reviewed in Nicchitta, 1998). Therefore, KDEL containing proteins are capable of escaping the ER but there has been no evidence of a KDEL protein except calreticulin localizing to an acidic granule compartment.

Perforin is a lethal protein that has the ability to bind to phospholipid membranes and form pore structures within the membrane upon exposure to low levels of calcium (Blumenthal *et al.*, 1984; Henkart *et al.*, 1984; Podack *et al.*, 1985; Masson *et al.*, 1990). The interaction between calreticulin and perforin is calcium dependent. That is, the two proteins interact only in the absence of calcium as determined by both immunoprecipitation and protein overlay experiments. It is very unlikely that calreticulin interacts with perforin within the ER environment. Within the ER the free calcium concentration is in the order of 200 μM (Montero *et al.*, 1995) which according to the results presented here would dissociate calreticulin and perforin. For the protection of the CTL producing perforin the pore forming protein is produced as an inactive precursor that travels through the ER (Uellner *et al.*, 1998). The trafficking mechanisms that result in perforin being localized to the cytolytic granules are unknown. However, it is known that when perforin is past the ER compartment, in the acidic granules, it is proteolytically processed into a potentially active form (Uellner *et al.*, 1998). This proteolytic processing removes 20 amino acids resulting in the exposure of perforin's C2 phospholipid binding domain (Uellner *et al.*, 1998). It is here in the granules that another protection mechanism needs to be in place to maintain perforin in an inactive conformation until the granule contents have been exocytosed towards a target cell to be killed. The acidic environment of the granules has been shown to favour interactions between perforin and the proteoglycans which would assist in preventing perforin from being activated. Also, the calcium concentration of these granules has been assumed to be low and it is this environment that would favour the interaction between calreticulin and perforin. It is plausible that calreticulin's role in the granules may be to help maintain perforin in an inactive conformation by direct protein-protein interaction. Whether or not calreticulin is acting like a chaperone for perforin in the granules is not clear at this point but from this work it is clear that calreticulin is unlikely to chaperone perforin as it travels from the ER to its granule location.

Using both the protein overlay technique and the two hybrid system it was determined that the interaction between calreticulin and perforin is highly dependent on the P-domain of calreticulin. The N-domain was also found to be involved in the interaction with the protein overlay technique but not the two hybrid system. The reason for the discrepancy is not clear. It may simply be due to the completely different environments in which the experiments are carried out but having the two hybrid experiments carried out within a cell would indicate the data is likely more physiologically relevant. The P-domain of calreticulin has several interesting features that make this finding significant. The P-domain appears to be of functional importance. It contains the high affinity calcium binding site ($K_m = 1\ \mu M$) (Baksh and Michalak, 1991). This domain contains two sets of three amino acid repeats (P-x-x-I-x-D-P-D-A-x-K-P-E-D-W-D-E and G-x-W-x-P-P-x-I-x-N-P-Y-x, respectively)

(Michalak ed., 1996) which are thought to be important in calreticulin's chaperone function (Vassilakos *et al.*,1998) as well as being involved in its high affinity calcium binding site. It is this region that shows considerable homology to another resident ER chaperone, calnexin. Calnexin and calreticulin are unique chaperones in that they are lectins that specifically recognize monoglucosylated, N-linked oligosaccharides (Hammond and Helenius, 1995; Helenius *et al.*, 1997). The P-domain of calreticulin requires calcium to bind to the monoglucosylated oligosaccharides (Vassilakos *et al.*,1998) which would indicate that the interaction between calreticulin and perforin is not dependent on perforin's glycosylation state. As well, mature perforin, present in the granules, contains only complex glycans (Uellner *et al.*, 1998) that are not recognized by calreticulin (Helenius *et al.*, 1997). The protein overlay experiment also confirms this because the radiolabeled perforin used in this experiment was not glycosylated yet clearly interacted with calreticulin.

It appears that the calreticulin-perforin interaction may be similar to the interaction between calreticulin and PDI (Baksh *et al.*, 1995). It was shown that calreticulin interacts via its N- and P-domains with PDI. In this case, calreticulin's interaction with PDI inhibits PDI activity and prevents calcium binding to calreticulin's high affinity calcium binding site (Baksh *et al.*, 1995). Therefore, this provides another example where calreticulin interacting with another protein, in the absence of calcium, has an inhibitory affect on the protein's function. Calreticulin-protein interactions appear to be of importance to calreticulin's function within the cell.

Intracellular calcium levels are increased in the stores of stimulated CTLs (Clementi *et al.*, 1994). CTL activation leads to an initial calcium release from internal stores followed by an influx of calcium across that plasma membrane (Gray *et al.*, 1987; Gray *et al.*, 1988; Haverstick *et al.*, 1991). It has been shown that calreticulin is involved in calcium storage in the ER (Bastianutto *et al.*, 1995; Mery *et al.*, 1996) and in the regulation of calcium influx across the plasma membrane in response to calcium store depletion (Mery *et al.*, 1996). The level of calreticulin in resting T-cells appears low and is significantly increased in stimulated CTLs (Burns *et al.*, 1992). Calreticulin is localized to the granules in CTLs (Dupuis *et al.*, 1993) which is confirmed by the GFP-calreticulin localization and immunostaining results of this study. However, this does not preclude the possibility that there is low levels of calreticulin still present in the ER that are not detectable under these experimental conditions. Therefore, calreticulin may be playing a role in calcium homeostasis and calcium signaling in the stimulated CTL. This would suggest a dual role for calreticulin in activated T-cells. Calreticulin may be involved in the regulation of the calcium signaling that is required for T-cell activation and it may be involved as a perforin "assistant" within the granules helping to maintain perforin in an inactive state until degranulation. This is beyond the scope of this calreticulin-perforin interaction study and would require further experimentation to address these questions.

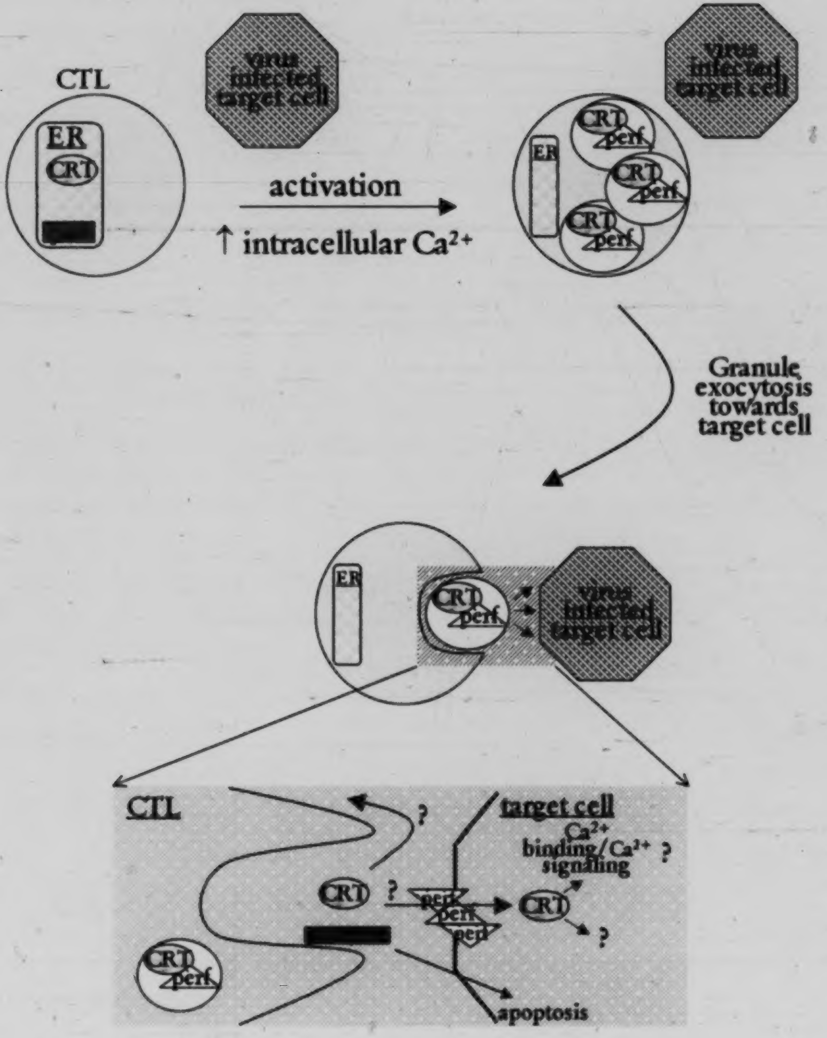
Calreticulin's localization to the granules may have a dual function in another way. As previously stated, its interaction with perforin may be involved in maintaining perforin in an inactive state before granule excocytosis. There is also evidence that calreticulin is released upon degranulation along with the other components of the cytolytic granules (Dupuis *et al*, 1993). This suggests that calreticulin may be involved in the killing of the target cell or possibly in a protective role for the CTL during target cell killing. As the granule contents are released towards the target cell the calreticulin-perforin complex would be exposed to much higher calcium concentrations which would favour dissociation of the two proteins. At this point extracellular calreticulin has the potential to then carry out another, separate function from that within the granule. Calreticulin has been previously reported in extracellular locations (Sueyoshi *et al.*, 1991; Eggleton *et al.*, 1994; Gray *et al.*, 1995; White *et al.*, 1995). Calreticulin has been found to be involved in inflammatory responses (Eggleton *et al.*, 1994) and acts as an antithrombotic agent (Kuwabara *et al.*, 1995). Extracellular calreticulin has been shown to be involved in several different protein-protein interactions including binding to fibrinogen (Gray et al., 1995) and binding to vitamin K-dependent coagulation factors where calreticulin inhibits experimentally induced coronary thrombosis (Kuwabara et al., 1995). Calreticulin may be able to bind to factors present on the surface of target cells or gain entry into the target cell cytoplasm where it may be able to alter gene expression and/or calcium homeostasis. Alternatively, calreticulin may be able to bind to the surface of the CTL after degranulation and prevent perforin from destroying the CTL. Either way, the potential exists that calreticulin may be a valuable player in cell-mediated target cell killing and determination of its exact functional role will require further study.

SUMMARY

Calreticulin's presence in the cytolytic granules of CTLs has been confirmed in this study. Importantly, the granule localized calreticulin was determined to contain an intact, C-terminal, KDEL ER retention/retrieval sequence indicating that calreticulin has the ability to escape the ER and may contain alternative targeting information. This is supported by the fact that calreticulin is localized to the CTL granules from perforin knockout mice and does not require an interaction with perforin for granule targeting to occur. It has been determined that calreticulin interacts directly with perforin in a calcium dependent manner as established by both immunoprecipitation and protein overlay experiments. This interaction appears to occur under conditions that would be favoured within the cytolytic granules. The calreticulin-perforin interaction is also highly dependent on the P-domain of calreticulin as determined under the *in vivo* conditions of the yeast two-hybrid system. This data all suggests that calreticulin is not simply acting as a chaperone for perforin as it travels through the ER for two reasons: 1) the unfavourable environment within the ER to support the interaction discovered here and 2) the fact that calreticulin is localized to the granules in the absence of calreticulin. This study also leads to the possibility that calreticulin is involved in maintaining perforin in an inactive state until granule exocytosis at which point calreticulin may play a role in target cell killing (Fig. 5-9). Calreticulin's exact functional role(s) in CTLs is still unclear and will require further study. The data presented here provides essential pieces to the puzzle and will aid in the interpretation of functional studies in the future.

Figure 5-9: Potential roles for calreticulin in CTL target cell killing

This is a schematic diagram illustrating the possible involvement of CRT in CTL mediated target cell killing. Perforin travels through the ER as an inactive precursor as indicated by the dark grey rectangle. CRT and perforin interaction is not favoured within the ER. Upon T-cell activation CRT and perforin both localize to the granules. Perforin is proteolytically processed to a potentially active for in this acidic compartment. The interaction between CRT and perforin is now favoured in this environment as shown and may be important for maintaining perforin in an inactive conformation. Upon granule exocytosis toward the target cell the granule contents are released where perforin inserts and polymerizes in the target cell membrane. CRT potential involvement upon degranulation is indicated by the question marks.


CTL
virus infected target cell
ER
CRT
activation
↑ intracellular Ca²⁺
virus infected target cell
CRT
perf.
ER
Granule exocytosis towards target cell
ER
CRT
perf.
virus infected target cell
CTL
?
target cell
Ca²⁺ binding/Ca²⁺ signaling
?
CRT
?
perf
perf
perf
CRT
?
CRT
perf.
apoptosis

CHAPTER SIX

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Major observations of my work are as follows 1) the production and purification of a glycosylated calreticulin from the *Pichia* protein expression system, 2) the unexpected determination that calreticulin most likely does not interact directly with the GR *in vivo* and the identification of calreticulin as a potential signaling molecule 3) the identification and characterization of the interaction between calreticulin and perforin as well as the determination that the alternate targeting of calreticulin to the granules is independent of any calreticulin-perforin interaction.

The production of calreticulin in a eukaryotic expression system that resulted in the glycosylation of the protein is of great importance. The role of glycosylation in calreticulin function has been both contradictory and elusive. The fact that the glycosylation of calreticulin is tissue and species dependent has meant that study of this aspect of the protein has been difficult. The data presented here indicates that the glycosylation site found in rabbit calreticulin is functional but the circumstances of glycosylation are not clear. Another fundamental result of this work is the production of P-domain specific anti-calreticulin antibodies. This indicates that the P-domain is the most antigenic part of the yeast calreticulin and the most exposed. P-domain specific antibodies have not been generated previously that we know of. The yeast protein behaves like the endogenous protein but may contain subtle differences in protein conformation.

The importance of the finding that calreticulin does not interact with the DNA binding domain of the GR *in vivo* and that it is the ER localized calreticulin that modulates steroid sensitive gene expression cannot be overrstated. This finding is in complete opposition to the pervious assumption that calreticulin modulated steroid-sensitive gene expression through direct interaction with the various hormone receptors. My results indicate that calreticulin's functional role in steroid sensitive gene expression is indirect. This discovery also provides the first solid data indicating that calreticulin may be a signaling molecule from the ER.

The interaction between calreticulin and perforin described here provides information about a novel putative function for calreticulin. The evidence suggests that calreticulin interacts with perforin within the granule environment implying a role for calreticulin as an assistant in maintaining perforin in an inactive state while localized within the CTL. My work also establishes that the environment during granule exocytosis would not be favourable for maintaining the calreticulin-perforin interaction. If calreticulin is no longer "assisting" perforin there remains the possibility that calreticulin could play a distinct role in target cell killing. Although further research is required to answer these questions my data suggests that calreticulin may have more than one function in CTLs. Another vital discovery is that calreticulin retains its KDEL ER retrieval sequence in the CTL granules. An unexpected finding is that calreticulin localizes to the granules in the absence of perforin. This indicates that calreticulin may contain targeting information that allows its escape from the ER lumen. Therefore, this data also provides unique insights into the intracellular targeting of calreticulin.

While examining calreticulin-protein interactions my work provided invaluable information about calreticulin's role in different cellular environments as well as provided tools and knowledge on which to base further study of the structure and function of calreticulin.

Glycosylation

The glycosylation of calreticulin is a confusing subject. Glycosylation of calreticulin appears to be both species and tissue dependent. How and why calreticulin is only glycosylated under certain conditions and in specific species are completely unknown.

Unexpectedly, in the process of producing calreticulin in the *Pichia* expression system the resulting protein was glycosylated. This project used the rabbit cDNA of calreticulin to generate a secreted calreticulin in yeast. Importantly, the native rabbit protein has not been found to be glycosylated. The glycosylation of calreticulin under these circumstances then raises several questions.

Several years ago an important cellular response to heat stress was noted and referred to as the "prompt glycosylation stress response" (Henle *et al.*, 1993). It was determined that in the earliest stages of the heat stress response there was a disruption of the constitutive glycosylation process which was replaced by the glycosylation of a select few proteins (Henle *et al.*, 1993). This prompt glycosylation was independent of protein synthesis and therefore appeared to be occurring on mature proteins. Of the few proteins calreticulin was identified as one of the major proteins glycosylated in this process (Jethmalani *et al.*, 1994). The reason for the cell's rapid glycosylation of a small set of mature proteins in response to stress situation is not clear. It has been suggested that prompt glycosylation may alter the interaction of chaperones with their potential protein substrates under the heat stress conditions (Henle *et al.*, 1993). Presumably, this would allow the cell to maintain the quality control of maturing proteins during disruptive, stress inducing conditions. An alternative hypothesis, which is not necessarily exclusive of the first, is that prompt glycosylation would confer stability to (Henle *et al.*, 1993) and possibly prevent aggregation (Jethmalani *et al.*, 1994) of the proteins required to handle the stress conditions. The increased structural and thermodynamic stability of proteins in response to glycosylation has been previously suggested (Henle ed., 1987). The prompt glycosylation response has also been suggested to alter protein-protein interactions (Henle *et al.*, 1993)

By analogy, the production of glycosylated calreticulin in the *Pichia* expression system may be due to a stress response. Although prompt glycosylation in response to stress conditions other than heat shock has not been identified it is plausible that other stress inducing circumstances could elicit a similar response. In this system, calreticulin is produced at significantly high levels that may in fact stress the ER by overloading it (reviewed in Pahl and Baeuerle, 1997). Another point worth noting is that yeast cells do not contain any endogenous calreticulin. The overproduction of any ER targeted protein could potentially result in aggregation, altered protein folding or stability but calreticulin, being a calcium binding protein and having chaperone capabilities, could also potentially have other far reaching affects within the ER. Glycosylation of calreticulin may then provide stability, prevent aggregation and allow the protein to traverse the secretion pathway in yeast without resulting in ER damage or congestion due to the protein's overexpression.

Although this is an interesting hypothesis as to why calreticulin is glycosylated under these circumstances it does not address the question of why or how the specific glycosylation of calreticulin occurs in other species/tissues that do not appear to be under any stress conditions. The yeast calreticulin then provides an unexpected tool for studying the functional implications of glycosylation of calreticulin. The stability of both glycosylated and unglycosylated calreticulin can be examined. Differences in the stability of calreticulin may be important under stress conditions but also in different tissues helping to explain the tissue specific glycosylation of the protein. As well, if the suggestion that prompt glycosylation may alter protein-protein interactions is correct it will be interesting to examine potential changes in calreticulin-protein interactions due to glycosylation. Preliminary analysis of the consequences of glycosylation of calreticulin using the yeast protein will be essential in the understanding of the specific nature of calreticulin glycosylation *in vivo*.

Calreticulin, ER signaling and modulation of steroid sensitive gene expression

The existence of ER to nucleus signaling in mammalian cells is only beginning to come into focus (reviewed in Pahl and Baeuerle, 1997). The finding in this study that calreticulin most likely does not interact directly with the GR in a physiological setting along with the fact that calreticulin localized to the ER is responsible for modulating GR sensitive gene expression is of profound importance. This is the first example that calreticulin can indirectly affect cellular processes from its location within the ER and provides a basis for examining calreticulin as a potential signaling molecule from the ER. Other examples of ER to nucleus signaling involves a complex array of protein-protein interactions to pass the signal from one cellular compartment to another. Most certainly calreticulin signaling will be no different. Is there a calreticulin receptor in the ER? Calcium plays a role in ER signaling involving NF-κB (Pahl and Baeuerle, 1997). Does calreticulin's calcium binding ability play a role in calreticulin signaling? It will be essential to identify calreticulin-protein interactions in the ER that affect calreticulin's ability to alter gene expression to fully understand this presumably novel signaling pathway. Although calreticulin is already known to interact with PDI and ERp57 in the ER (Baksh *et al.*, 1995; Corbett *et al.*, 1998) a plausible involvement of these protein-protein interactions is not evident.

Calreticulin and chaperone function

Several studies have now established calreticulin as a molecular chaperone like its membrane bound homologue calnexin. The two chaperones are unique in that they are lectins that bind newly synthesized glycoproteins in the ER and assist in their maturation. There is evidence mounting that the binding of calreticulin and calnexin to nascent glycoproteins is not due to interaction with the monoglucosylated oligosaccharide chain alone (Arunachalam and Cresswell, 1995; Ware *et al.*, 1995; Williams, 1995; Zhang *et al.*, 1995; van Leeuwen and Kearse, 1996). There is a theory that the binding of these chaperones involves a two step process of recognition of the monoglucosylated glycan followed by stabilization of the interaction via protein-protein interactions (Ware *et al*, 1995; Zhang *et al.*, 1995). This theory is supported by recent work that demonstrates the oligosaccharide binding to the two chaperones to be the same and both proteins require calcium for the binding of the sugar to occur (Vassilakos *et al.*, 1998). Therefore, any difference in substrate specificity between the two chaperones may be due to interactions with the polypeptide chain. This is then another example of the importance of calreticulin-protein interactions.

I demonstrated that calreticulin interacts with PDI. This protein-protein interaction inhibits PDI's ability to refold scrambled RNAse B (Baksh *et al.*, 1995). The interaction is calcium dependent (Baksh *et al.*, 1995). With the data available regarding calreticulin's chaperone ability this suggests that calreticulin may play a complex, integral role in the maturation of proteins in the ER. In the low calcium conditions of the ER when the stores have been depleted calreticulin and PDI would interact possibly preventing either of the two proteins from interacting with nascent polypeptides. Upon refilling of the calcium stores calreticulin and PDI would disassociate and be allowed to carry out their respective roles in the maturation of nascent proteins in the ER. A very recent study has indicated that in the presence of calcium the interaction between calreticulin and ERp57, another ER chaperone with PDI like activity, is stabilized (Corbett *et al.*, 1998). Therefore, under increased calcium conditions, calreticulin would be released from PDI and be free to bind ERp57 in complex along with newly synthesized glycoproteins. This complex formation would then maintain the glycoprotein in the ER while ERp57 had the opportunity to assist in its folding (Corbett *et al.*, 1998). The calreticulin-PDI interaction then adds another piece to the complexity of calreticulin's role in the quality control of glycoproteins traveling through the ER.

The work presented here also identified a calcium dependent interaction between calreticulin and perforin. Previous work co-localizing calreticulin and perforin to the CTL granules had suggested that calreticulin may be acting as a molecular chaperone for perforin (Dupuis *et al.*, 1993). Several indications made this theory plausible. To protect the CTL perforin must be maintained in an inactive state and be sequestered away from calcium to prevent activation and polymerization of the perforin into pore structures that would cause lysis of the CTL. Calreticulin, acting as a molecular chaperone for newly synthesized glycoproteins and being able to bind calcium would then be a good candidate for binding to perforin, accompanying it to the granules and sequestering calcium. This is not the case. Calreticulin's ability to interact with perforin is an example of a calreticulin-protein interaction that is likely separate from its molecular chaperoning function in the ER for several reasons. Calreticulin and perforin interact only in the absence of calcium and calreticulin's chaperone function requires calcium (Vassilakos *et al.*, 1998). Calreticulin is targeted to the granules in the absence of perforin and therefore does not "tag along" with perforin from the ER. Also in support of this data is the finding that perforin is synthesized as an inactive precursor in the ER and is only processed to a potentially active form after its arrival in a downstream acidic compartment (Uellner et al., 1997) therefore would not need to interact with calreticulin in the ER to maintain an inactive conformation. Importantly, although this work does not dispute calreticulin's ability to act as a molecular chaperone calreticulin's interaction with perforin is presumed to be functionally distinct.

Calreticulin and target cell killing

Examination of calreticulin's potential role in CTL mediated target cell killing will be essential to understanding how this calcium binding protein may be involved beyond its interaction with perforin in the granules. It will be important to address the question as to where does calreticulin go after the granule contents are exocytosed towards the target cell. Does calreticulin enter the target cell? Does calreticulin bind to surface molecules of the CTL? The availability of GFP-calreticulin constructs may provide clues as to where calreticulin is located after degranulation. Another consideration to be made is that due to the fact that calreticulin co-purifies with perforin it is possible that some of the studies done with purified perforin may have been done unknowingly in the presence of calreticulin. Results that were attributed to perforin alone may of in fact have been due in part to the presence of calreticulin. Examination of perforin's killing ability in the presence and absence of calreticulin should likely be clarified.

Localization of calreticulin

Throughout this study fundamental information about calreticulin's ability to localize to different subcellular locations, with its KDEL retrieval sequence intact, has been uncovered. Not surprisingly, calreticulin is generally accepted to be a resident lumenal ER protein with its N-terminal signal sequence and its C-terminal KDEL retrieval sequence. Specific studies of the mechanisms involved in maintaining calreticulin in the ER revealed the functional importance of its C-terminal KDEL retrieval sequence. In one case of calreticulin in rat hepatocytes it was found that calreticulin does in fact leave the ER to traverse through the Golgi apparatus (Peter *et al.*, 1992). This study demonstrated that the majority of calreticulin left the ER and traveled to the Golgi where it was then retrieved by the KDEL receptor and taken back to the ER (Peter *et al.*, 1992). It was later determined that calreticulin is also retained in the ER through a KDEL independent mechanism in that it requires the C-domain of the protein (Sönnichsen *et al.*, 1994). A very small percentage of calreticulin was found to be secreted into the media which was increased by mutation of calreticulin by deleting the C-domain but retaining the KDEL retrieval sequence (Sönnichsen *et al.*, 1994). This then provided preliminary evidence that even with an intact KDEL retrieval sequence calreticulin could in fact escape the ER. This finding was not unique to calreticulin. The KDEL containing proteins, PDI, BiP and GRP94 have all been detected on the cell surface in small quantities (Yoshimori *et al.*, 1990; Altmeyer *et al.*, 1996).

Yeast calreticulin is efficiently secreted with its KDEL retrieval sequence intact. The reason why calreticulin is not retrieved to the ER in yeast in not clear. The α factor signal sequence used in the production of calreticulin in yeast is presumably responsible for the efficient secretion of calreticulin. How can a signal sequence that is ultimately cleaved from the protein cause the secretion of a protein with an intact ER retrieval sequence? Is the level of calreticulin overexpression simply beyond the capability of the KDEL retrieval system? The explanation is not likely simple due to the fact that overexpression of calreticulin has not been found to alter its localization in previous studies (Sönnichsen *et al.*, 1994). The fact that other KDEL containing proteins have been detected on the cell surface in small quantities suggests the possibility that an alternative, albeit minor, targeting pathway may exist for KDEL proteins.

Calreticulin in CTL's also manages to escape the ER with its KDEL retrieval sequence intact. This localization is independent of calreticulin's interaction with perforin. Therefore, this interaction is not due to masking of the KDEL sequence from the KDEL receptor. Although other KDEL proteins escape the ER, calreticulin is the only KDEL protein that is localized to the granules. Many proteins located in the granules arrive there due to the mannose-6-phosphate receptor pathway known to target proteins that contain mannose-6-phosphate modified glycans to lysosomal/endosomal compartments (reviewed in Griffiths and Argon, 1995 and Griffiths, 1997). Calreticulin does not contain the modified glycan and therefore does not utilize this pathway for targeting to the granules. What specific feature of calreticulin then allows for this localization? An appealing possibility is the presence of two lysosomal targeting sequences within calreticulin. These have not been shown to be functional, nor has calreticulin ever been found in a lysosomal compartment in other cells. Nevertheless, it will be essential to examine these targeting signals, possibly by mutagenesis, to determine whether or not they are recognized by the protein sorting machinery in CTLs. As well, to understand more about calreticulin trafficking it will be essential to identify the differences in protein sorting mechanisms in CTLs as opposed to other cells. Are there specific protein-protein interactions occurring with calreticulin in CTLs that allow for the packaging of calreticulin into granules?

General Comments

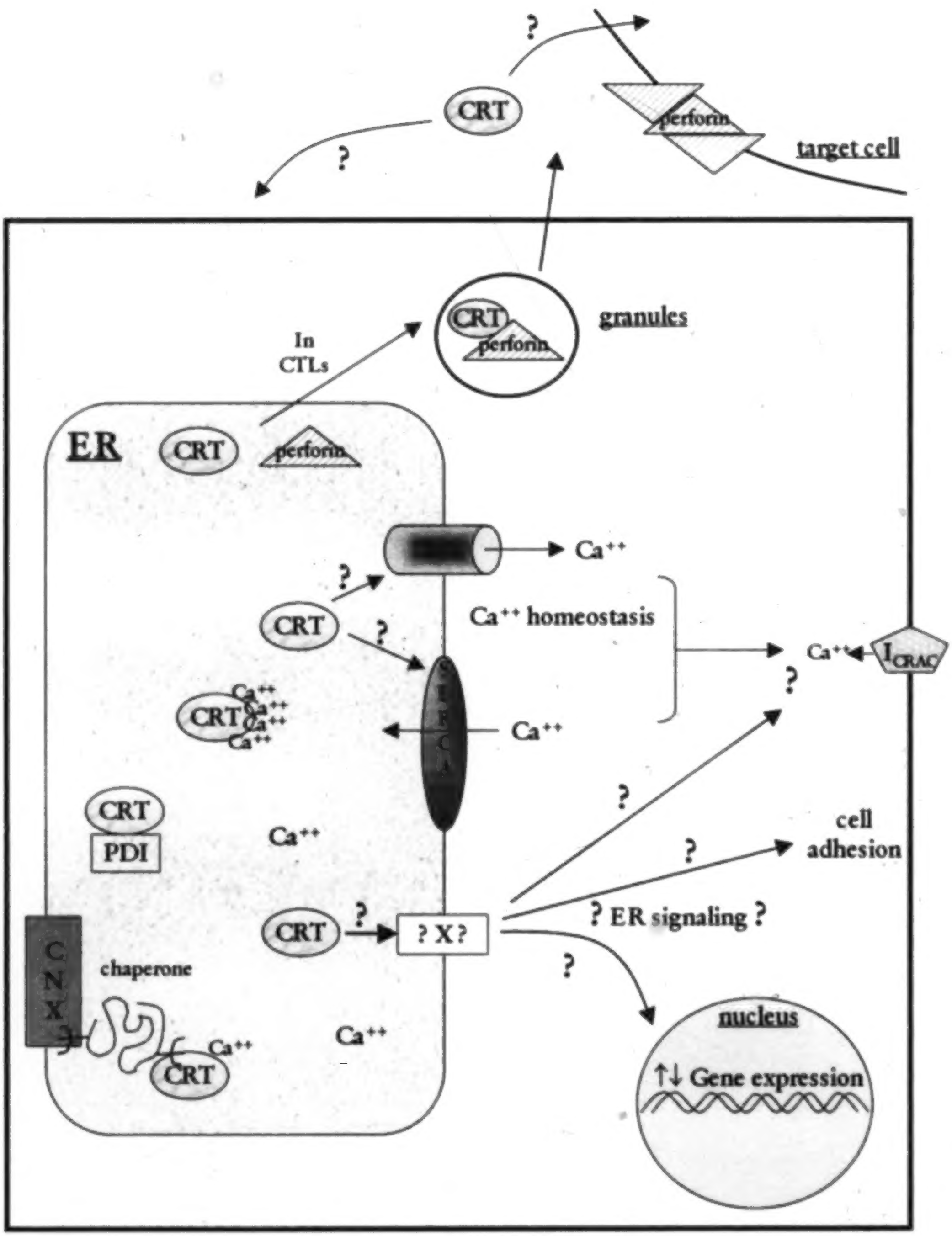
Protein-protein interactions are essential to virtually every cellular process and pathway (for review see Phizicky and Fields, 1995 and references therein). Protein-protein interactions are involved in DNA replication, transcription and translation. They regulate gene transcription, cell proliferation, all aspects of metabolism and signal transduction. Protein-protein interactions can affect processes in many different ways. They may cause changes in conformation, they may cause changes in the kinetic properties of a protein or they may create a new binding site for a substrate. Protein-protein interactions can inhibit function or enhance function of a protein. They are a fundamental part of cell survival and cell death. Understanding protein-protein interactions occurring between specific molecules allows for an understanding of the mechanisms involved in a given cellular process.

Therefore, the study of protein-protein interactions provides insight into the functional role that a protein may play within the cell.

Calreticulin is a fascinating protein that appears to affect cellular functions in several regions around the cell (Fig. 6-1). The answers to the remaining questions surrounding the functional roles that calreticulin plays will likely come from all different areas of biological research. It is evident that calreticulin's ability to act as a multifunctional protein is likely through its protein-protein interactions throughout the cell. The identification of calreticulin's protein partners will be an important part of calreticulin research in the future. Understanding the nature of all these protein interactions will help to put all the pieces of the calreticulin functional puzzle in place. With the information about calreticulin to date it seems plausible that dysfunction in any of calreticulin's functional roles in the cell could have clinical implications. Once our understanding of how calreticulin functions in all its different roles is more complete we can start to examine the possibility of manipulating calreticulin to our advantage. These thoughts are far reaching down the road of calreticulin research and there will likely be much debate and controversy yet to come surrounding this unusual and interesting protein.

Figure 6-1: Model of calreticulin's possible actions in the cell

This is a schematic overview of all the different potential roles CRT may be involved in within different cell types. The potential involvement of CRT in CTL granules (upper portion of the diagram) and ER signaling (lower right hand portion of the diagram) that pertain to this study are shown. CRT's role as an ER chaperone and its likely involvement in calcium homeostasis are also briefly depicted.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CNX
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CRT
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nucleus
↑↓ Gene expression

CHAPTER SEVEN

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